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13. ABSTRACT ***(Maximum 200 Words)***

Clinical and experimental evidence suggest that the insulin-like growth factor receptor type 1 (IGF-IR) is involved in breast cancer etiology. IGF-IR is overexpressed in breast tumors (relative to normal breast epithelium) and high levels of IGF-I (IGF-IR ligand) correlate with breast cancer risk in premenopausal women. In vitro, IGF-IR regulates breast cancer proliferation and survival. In addition, we and others have shown that IGF-IR signaling plays a role in the development of estrogen-independence and antiestrogen-resistance. It is now becoming evident that the activation of mitogenic and anti-apoptotic functions of IGF-IR may be critical in primary tumor growth and the early stage of tumorigenesis, however, the involvement of IGF-IR in growth-unrelated processes which may regulate cell spread and cancer metastasis (cell adhesion, motility, chemoattraction) is not clear. To address this problem, we developed several cell lines expressing IGF-IR mutants. The clones will be used to map signaling pathways involved in intercellular adhesion, migration and chemoattraction. The future work will extend our findings reported here that breast cancer cell adhesion and migration on fibronectin involves a dynamic interaction between an IGF-IR substrate SHC and alpha5beta1 integrin.

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INTRODUCTION

Clinical and experimental evidence strongly suggest a role of IGF-IR in breast cancer etiology and progression (1, 2). In particular, IGF-IR is overexpressed in breast cancer compared with its levels in normal epithelial cells or in benign breast tumors (3-5) and IGF-IR ligands (IGF-I and IGF-II) are potent mitogens for breast cancer cells in culture and have been detected in the epithelial and/or stromal component of breast tumors (6). In addition, a strong correlation between circulating levels of IGF-I and breast cancer risk in premenopausal women has been recently reported (7). Moreover, high levels of IGF-IR or its major substrate IRS-1 correlated with cancer recurrence at the primary site (4, 8). Besides promoting cell growth, IGF-IR, especially the IRS-1 pathway, protects breast cancer cells from apoptosis induced by different anti-tumor drugs or by low concentrations of mitogens (growth factors and steroid hormones) (9-13).

Although ample evidence suggests that abnormal activation of IGF-IR may contribute to the autonomous growth and increased survival of breast tumor cells at the primary site, the function of this receptor in breast cancer metastasis is not clear. In this respect, IGF-IR expression has been described as either positive or non-significant marker of overall survival. Some small clinical studies demonstrated a correlation between IGF-IR expression in node-positive tumors and worse prognosis (1). Other studies linked IGF-IR expression with better prognosis as IGF-IR was predominantly expressed in a subset of breast tumors with good prognostic characteristics (1, 5). In the experimental setting, IGF-I stimulates cell motility in MCF-7 and MDA-MB-231 cells on collagen, which may suggest a role of the IGF-IR in cell spread (14).

The long-term goal of this project has been to understand the role of IGF-IR and its various signaling pathways in the development and neoplastic progression of breast tumors. The following aims have been planned for Year 3:

(a) Continue characterization of MCF-7 cells expressing IGF-IR mutants with emphasis on transforming potential, cell-cell adhesion and survival in 3-D culture.
(b) Continue characterization of MCF-7/SHC cell lines focusing on mitogenicity, transforming abilities, motility, and survival.

In this report period, we set on examining the importance of IGF-IR signaling in cell adhesion and motility. We approached the problem by generating cell lines expressing either different elements of the IGF-IR pathway or different signaling mutants of IGF-IR. In the first aim, our focus was on an IGF-IR signaling substrate SHC, because this molecule, unlike the other IGF-IR substrate IRS-1, plays little role in IGF-I-dependent growth. Regarding the second aim, we generated MCF-7 breast cancer cells in which either all or only certain IGF-IR pathways have been blocked.

TECHNICAL REPORT

The experiments proceeded according to the Statement of Work.

(a) Development and characterization of MCF-7 cell lines expressing mutants of the IGF-IR.

The involvement of different signaling pathways in controlling the phenotype of breast cancer cells can be studied in cells expressing dominant negative mutants of IGF-IR. Mutant receptors, through dimerization with normal receptors, block certain receptor domains, which interferes with the activation of different signaling pathways. To investigate which IGF-IR signals regulate different IGF-dependent functions in breast cancer cells, we developed MCF-7 cell lines expressing the following IGF-IR mutants: IGF-IR/KA, IGF-IR/KR and IGF-IR/TC. The first two mutants are "dead receptors" which act as dominant negative receptors inhibiting tyrosine kinase of IGF-IR wild-type. The TC mutant lacks the last C-terminal 108 aa of the beta-subunit of IGF-IR and its expression is expected to block only certain IGF-IR functions (e.g., transformation) without affecting others (e.g., mitogenesis) (15).

We developed several cell lines expressing mutant IGF-IRs by stable transfection with pcDNA3-based vectors encoding the relevant IGF-IR cDNA, followed by selection in G418. The level of IGF-IR expression was analyzed by FACS analysis and confirmed by Western blotting, as described before (11, 15, Fig. 1). For further analysis, we selected one representative clone of each group. The clones expressing different mutants have been named: MCF-7/IGF-IR/KA, MCF-7/IGF-IR/KR, and MCF-7/IGF-IR/TC cells (see top panels Fig.1).

Fig. 2 demonstrates the activation of IGF-dependent pathways in the above cell lines. We found that overexpression of "dead" IGF-IR mutants containing either an inactive (IGF-IR/KR) or significantly impaired (IGF-IR/3Y) tyrosine kinase abrogated or dramatically reduced several IGF-IR activities, such as IGF-IR autophosphorylation, IGF-dependent tyrosine phosphorylation of IRS-1, IRS-1/PI-3K binding and MAPK activation (Fig. 2). Please note that the levels of IGF-IRs in the cells expressing "dead" mutants are higher that that in MCF-7/IGF-IR clone ($1.1x10^6$), which indicates that the absence of signal was not due to a low receptor expression.

With MCF-7/IGF-I/TC cells, we found that the IGF-IR/IRS-1/PI-3 kinase signal and MAP kinase activation are retained, but basal and IGF-I-dependent SHC tyrosine phosphorylation is inhibited.

The monolayer growth of MCF-7 cells carrying IGF-IR/KA or IGF-IR/KA was ~30% inhibited compared with cells expressing the wild-type IGF-IR (control), while that of MCF-7/IGF-IR/TC was similar to control.

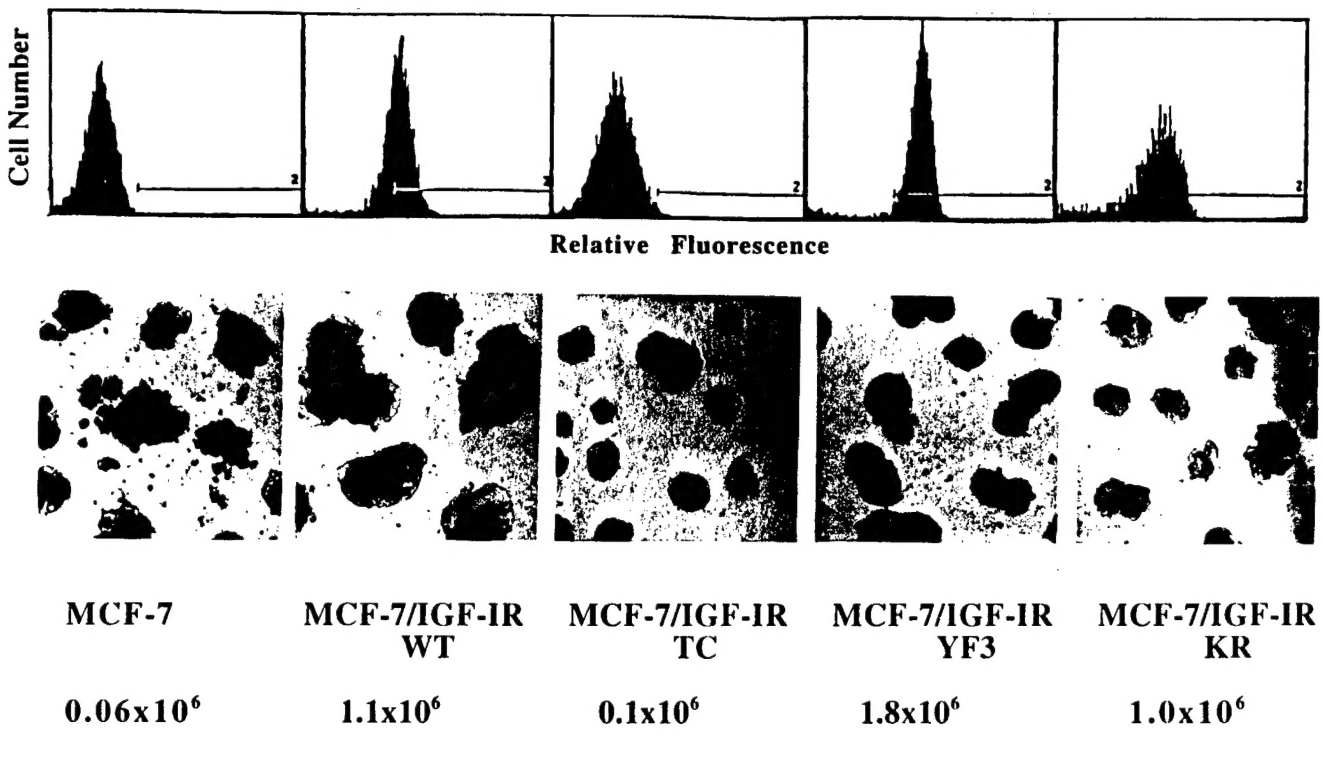

Fig.1. Breast cancer cell lines expressing IGF-IR mutants. *MCF-7 cell lines expressing IGF-IR/KA, IGF-IR/KR and IGF-IR/TC were developed using methodology described in Ref. . MCF-7 and MCF-7/IGF-IR (overexpressing a wild-type IGF-IR) were used as controls. Top panels, the levels of IGF-IR expression by FACS analysis with an anti-IGF-IR monoclonal antibody/anti-mouse fluorescein-conjugated IgG. Bottom panels, IGF-IR number in each cell line. Middle panels, the size of cell aggregates formed by different cell lines in 3-D culture on Matrigel (technique described by us before in Ref.11).*

The obtained cell lines will serve to study which IGF signaling pathway is required for cell-cell adhesion and/or migration. We have already performed preliminary experiments examining IGF-IR-dependent cell-cell adhesion in the developed mutants and found that this function of the receptor is inhibited in MCF-7/IGF-IR/KR, MCF-7/IGF-IR/KA and MCF-7/IGF-IR/TC cell lines (Fig. 2, bottom panel). This suggests that the stimulation of cell-cell adhesion requires tyrosine kinase activity of IGF-IR as well as the activation of a signal emanating from the C-terminus. The role of these pathways in cell motility will be pursued in the future.

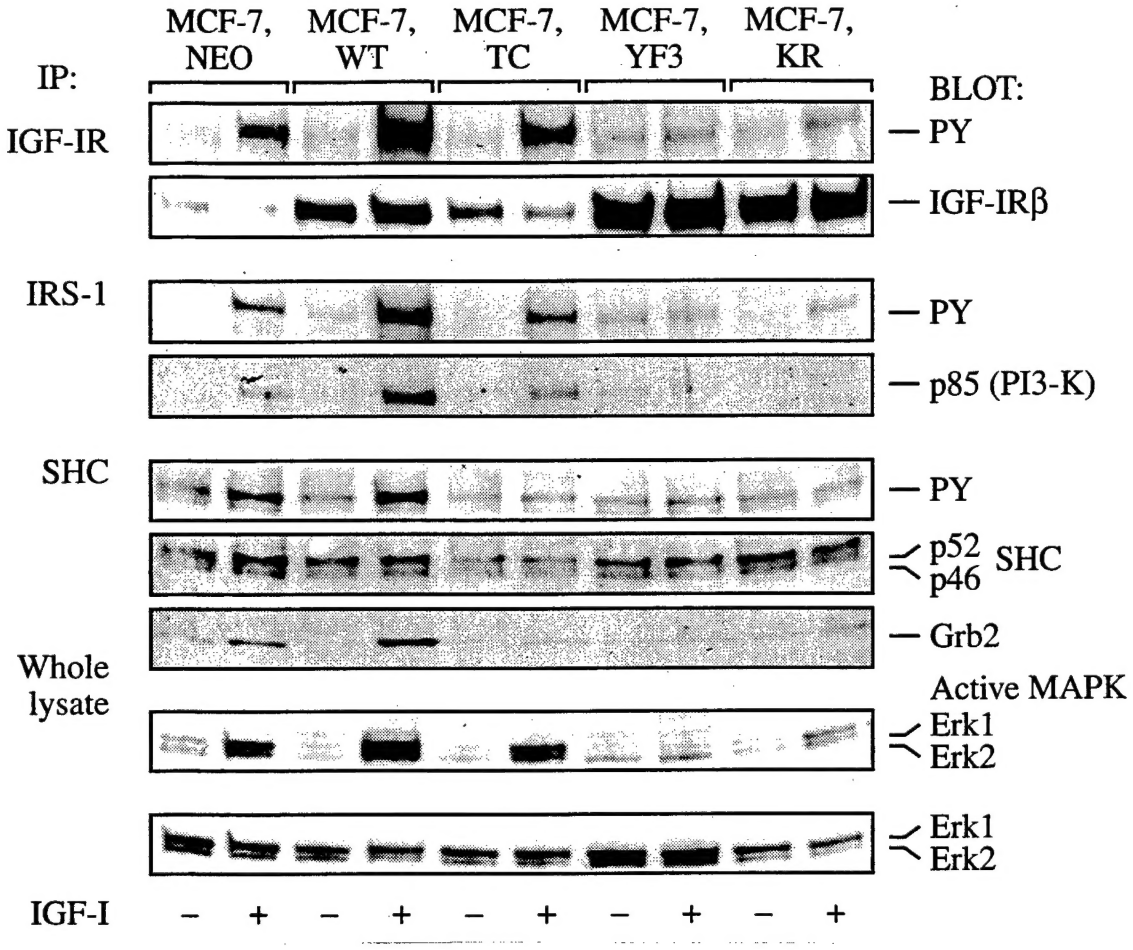

Fig. 1. IGF-IR signaling in MCF-7/IGF-IR/TC, MCF-7/IGF-IR/3Y and MCF-7/IGF-IR/KR cells. *The cells expressing dominant-negative mutants of the IGF-IR were generated by stable transfection of MCF-7 cells with either pcDNA3/IGF-IR/TC, pcDNA3/IGF-IR/KR or pcDNA3/IGF-IR/3Y encoding the following IGF-IR mutants, respectively: an IGF-IR with a truncation of the C-terminus (108 aa), an IGF-IR with an inactivated ATP binding site in the TK domain, and an IGF-IR with Tyr 1131, 1135 and 1136 in the TK domain replaced with Phe. MCF, NEO are MCF-7 cells stably expressing an empty vector (negative control) and MCF-7, WT is MCF-7/IGF-IR, clone 17, overexpressing a wild-type IGF-IR (Fig. 1)(positive control, Ref. 11). The above clones were identified by FACS analysis, and then by Western blotting, as described in Ref.11. All cell lines were synchronized in PRF-SFM for 24 h and stimulated with 50 ng/ml IGF for 10 min. The IGF-IR, IRS-1 and SHC were immunoprecipitated from 200 ug of protein lysate with an anti-IGF-IR mAb (Oncogene Science), an anti-IRS-1 pAb (UBI), and anti-SHC pAb (Transduction Laboratories), respectively. The levels of the IGF-IR were measured by Western blotting with an anti-IGF-IR pAb (Santa Cruz). The amounts of IRS-1-associated PI-3K were determined with an anti-p85 mAb (UBI). The levels of SHC were evaluated with an-anti-SHC mAb (Transduction Laboratories). The levels of SHC-associated GRB-2 were probed with an anti-GRB 2 mAb (Transduction Laboratories). Tyrosine phosphorylation of the IGF-IR, IRS-1 and SHC was determined using an anti-phosphotyrosine mAb PY20 (Transduction Laboratories). The activity and protein levels of MAPK (ERK1 and ERK2) were measured by Western blotting in 50 ug of total lysate with an anti-active MAP mAb (Promega) and an anti-ERK1 mAb (Transduction Laboratories), respectively. The results for one representative clone of each group are shown.*

(b) Development and characterization of MCF-7 cell lines overexpressing SHC.

The oncogenic SHC proteins (SH2 homology/collagen homology proteins p47, p52, and p66) are signaling substrates for most receptor and cytoplasmic tyrosine kinases (TKs) and have been implicated in cellular growth, transformation and differentiation. In tumor cells overexpressing TKs, the levels of tyrosine phosphorylated SHC are chronically elevated (16). The significance of amplified SHC signaling in breast tumorigenesis and metastasis remains unknown.

Recent experimental evidence indicates that SHC is involved in the regulation of cell adhesion and motility of many different cell types. For instance, overexpressed SHC mediated scattering through the NGF receptor in MDCK cells, and downregulation of SHC reduced EGF-dependent motility in MCF-7 breast cancer cells (10, 17). SHC has been found coupled with a class of extracellular matrix (ECM) receptors, specifically with integrins alpha1beta1, alpha5beta1, alphavbeta3, and alpha6beta4 (but not with alpha2beta1, alpha3beta1, and alpha6beta1) (18). Association of SHC with integrins is induced by the ligation of an integrin to an ECM substrate and leads to tyrosine phosphorylation of SHC, followed by SHC/GRB2-SOS binding and activation of Ras/MAP pathway, which in consequence may contribute to intracellular pathways regulating growth and survival, and perhaps migration (18). SHC is hyperactiveted in breast cancer (16) but the biologic significance of this phenomenon is not clear.

To address this problem, we developed breast cancer cell lines with a 7-9-fold overexpression of SHC over the levels in the parental cells (Fig. 1A and B, Mauro et al., in press, see Appendix). We demonstrated that the elevated levels of SHC did not significantly alter cell growth in monolayer or anchorage-independent culture (Fig. 1C and Tab. 1, Mauro et al., in press). However, high levels of SHC greatly improved interactions of cells with fibronectin (FN). Specifically, in human breast cancer cells overexpressing SHC (MCF-7/SHC) the association of SHC with alpha5beta1 integrin (FN receptor) was increased, spreading on FN accelerated, and basal growth on FN reduced (Fig. 2, 3 and Tab. 2 and 3, Mauro et al., in press). These effects coincided with an early decline of adhesion-dependent MAP (mitogen-activated) kinase activity (Fig. 4, Mauro et al., in press).

We also found that basal motility of MCF-7/SHC cells on FN was inhibited relative to that seen in several cell lines with normal SHC levels. However, when EGF or IGF-I were used as chemoattractants, the locomotion of MCF-7/SHC cells was greatly (~5-fold) stimulated, while it was only minimally altered in the control cells (Fig. 5, Mauro et al., in press).

These results, for the first time, point to the role of SHC in the regulation of cell adhesion and motility on FN in breast cancer cells (Mauro et al. in press in Exp. Cell Research).

Key Research Accomplishments:

- Demonstrated, for the first time, the function of a signaling molecule SHC in breast cancer cell adhesion and motility on fibronectin .
- Developed and partially characterized MCF-7 cell lines expressing different mutants of the IGF-IR.

Reportable Outcomes:

1. Manuscripts, abstracts and presentations:

Manuscripts:

- Mauro, L., Sisci, D., Bartucci, M., Salerno, M., Kim, J., Tam, T., Guvakova, M., Ando', S., Surmacz, E. SHC-alpha5beta1 integrin interactions regulate breast cancer cell adhesion and motility. Exp. Cell Res., in press, 1999.
- Guvakova, M., Surmacz, E. IGF-IR stimulates breast epithelial cell motility via reorganization of the actin cytoskeleton, remodeling of focal contacts, and modulation of the phosphorylation status of focal adhesion proteins: FAK, Cas, and paxillin.Exp. Cell Res., in press, 1999.
- Salerno, M., Sisci, D., Mauro, L., Guvakova, M., Ando', S., Surmacz, E. Insulin receptor substrate 1 (IRS-1) is a target of a pure antiestrogen ICI 182,780 in breast cancer cells. Int. J. Cancer, 81: 299-304, 1999.
- Ando', S., Panno, M. L., Salerno, M., Sisci, D., Mauro, L., Lanzino, M., Surmacz, E. Role of IRS-1 signaling in insulin-induced modulation of estrogen receptors in breast cancer cells. Biochem. Biophys. Res. Com., 253: 315-319, 1998.
- Surmacz, E. Function of the IGF-IR in breast cancer. J. Mamm. Gland Biol. Neoplasm. Submitted

Abstracts:

- Guvakova, M., Surmacz, E. Tyrosine kinase activity of the IGF-IR is required for the development of breast cancer cell aggregates in three-dimensional culture. AACR Annual Meeting, Philadelphia, PA, April 10-14, 1999
- Guvakova, M., Surmacz, E. IGF-IR tyrosine kinase is required for breast cancer epithelial cell motility. Specificity in Signal Transduction. Keystone Symp.Keystone, CO, April 9-14, 1999
- Mauro, L., Sisci, D., Salerno, M., Ando', S., Surmacz, E. Role of SHC signaling in breast cancer cell adhesion and motility. 21st Annual Breast Cancer Symposium, San Antonio, TX, December 12-15, 1998
- Guvakova, M., Surmacz, E . IGF-IR stimulates breast epithelial cell motility via reorganization of the actin cytoskeleton, remodeling of focal contacts, and modulation of the phosphorylation status of focal adhesion proteins: FAK, Cas, and paxillin.14th Annual Symposium on Cellular Endocrinology, Lake Placid, NY, September 24-27, 1998

Talks

- Surmacz, E. Is IGF-IR receptor involved in breast cancer? Staff Seminar Series, Kimmel Cancer Institute, Philadelphia, PA, January 18, 1999
- Surmacz, E. IGF and breast cancer. Prolactin and Growth Hormone: Mechanism and Function. International Hannah Symposium, Ayr, Scotland, UK, September 9-11, 1998 (invited speaker)

2. Patents and licences: None

3. Degrees: N/A

4. Development of biologic reagents:

Several new cell lines have been developed: MCF-7 cells expressing dominant negative mutants of the IGF-IR: MCF-7/KR and MCF-7/KA; MCF-7 cells expressing a signaling molecule SHC, MCF-7/SHC; metastatic breast cancer cell lines expressing IGF-IR, MDA-MB-231/IGF-IR.

5. Databases: None

6. Funding applied for:

- US Army Breast Cancer Research Program. "IGF-IR, Cell-cell Adhesion and Metastasis" 1999-2002. E. Surmacz, P.I., Founded.
- Susan G. Komen Breast Cancer Fundation, "IGF-I-dependent Antiestrogen Resistance in 3-D Culture". E. Surmacz, P.I. Pending.
- UICC International Union Against Cancer, "IGF-I-dependent Antiestrogen Resistance in 3-D Culture", Short-term Fellowship for Dr. Michele Salerno, E. Surmacz, Program Director, 1998. Founded.
- UICC International Union Against Cancer, "IGF-IR-mediated cell-cell adhesion", Short-term Fellowship for Dr. Loredana Mauro, E. Surmacz, Program Director, 1999. Founded.

7. Employment applied for: None

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SHC-α5β1 INTEGRIN INTERACTIONS REGULATE BREAST CANCER CELL ADHESION AND MOTILITY

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ABSTRACT

The oncogenic SHC proteins are signaling substrates for most receptor and cytoplasmic tyrosine kinases (TKs) and have been implicated in cellular growth, transformation and differentiation. In tumor cells overexpressing TKs, the levels of tyrosine phosphorylated SHC are chronically elevated. The significance of amplified SHC signaling in breast tumorigenesis and metastasis remains unknown. Here we demonstrate that 7-9-fold overexpression of SHC significantly altered interactions of cells with fibronectin (FN). Specifically, in human breast cancer cells overexpressing SHC (MCF-7/SHC) the association of SHC with α5β1 integrin (FN receptor) was increased, spreading on FN accelerated, and basal growth on FN reduced. These effects coincided with an early decline of adhesion-dependent MAP kinase activity. Basal motility of MCF-7/SHC cells on FN was inhibited relative to that in several cell lines with normal SHC levels. However, when EGF or IGF-I were used as chemoattractants, the locomotion of MCF-7/SHC cells was greatly (~5-fold) stimulated, while it was only minimally altered in the control cells. These data suggest that SHC is a mediator of the dynamic regulation of cell adhesion and motility on FN in breast cancer cells.

Key words: SHC, α5β1 integrin, fibronectin, motility, breast cancer

INTRODUCTION

The ubiquitous SH2 homology/collagen homology (SHC) proteins (p46, p52, and p66) are overlapping SH2-PTB adapter proteins that are targets and downstream effectors of most transmembrane and cytoplasmic tyrosine kinases (TKs) [1, 2]. Consequently, overexpression of $p52^{SHC}$ and $p46^{SHC}$ (referred to as SHC hereinafter) amplifies various cellular responses; for instance, induces mitogenic effects of growth factors in NIH 3T3 mouse fibroblasts and myeloid cells [1, 3], stimulates differentiation in PC12 rat pheochromocytoma [4], and augments hepatocyte growth factor (HGF)-induced proliferation and migration in A549 human lung adenocarcinoma [5]. Overexpressed SHC is oncogenic in NIH 3T3 mouse fibroblasts, but amplification of $p66^{SHC}$ isoform does not induce transformation [1, 6, 7] and may even inhibit growth pathways [8]. Importantly, increased tyrosine phosphorylation of SHC, which has been noticed in different tumor cell lines, is a marker of receptor or cytoplasmic TKs overexpression [2]. In breast cancer, for instance, SHC is hyperphosphorylated in cells overexpressing ERB-2 and c-Src [9, 10]. Whether such amplification of SHC signaling contributes to the development of a more aggressive phenotype of breast tumor cells remains unknown.

The effector pathways downstream of SHC are partially known. Upon tyrosine phosphorylation by TKs, SHC associates with the GRB2/SOS complex and subsequently stimulates the canonical Ras-MAPK (p42 and p44 Mitogen-Activated Protein Kinases) signal transduction cascade [1, 6, 7]. SHC/GRB2 binding and the activation of Ras are prerequisites for SHC-induced mitogenesis and transformation in NIH mouse fibroblasts [6]. In addition, SHC has been described to associate with adapters Crk II [11] and GRB7 [12], a signaling protein p145 [13, 14], and PEST tyrosine phosphatase [15] in various experimental systems. However, SHC pathways incorporating these signaling intermediates and their biological significance are not well understood.

There is substantial evidence suggesting that in addition to its role in mitogenesis and transformation, SHC also regulates non-growth processes, such as cell adhesion and

motility. For instance, overexpressed SHC improved motility in HGF-stimulated melanoma cells [5], and downregulation of SHC reduced epidermal growth factor (EGF)-dependent migration in MCF-7 breast cancer cells [16]. SHC was also essential for kidney epithelial cells scattering mediated by the receptors c-met, c-ros, and c-neu [17]. The mechanisms by which SHC regulates cell adhesion and motility are still obscure.

In several cell types (Jurkat, HUVEC, MG-63 and A431 cells), SHC couples with certain ECM receptors, specifically with the integrins $\alpha1\beta1$, $\alpha5\beta1$, $\alpha v\beta3$, and $\alpha6\beta4$, but not with $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha6\beta1$ [18]. Association of SHC with integrins may result in phosphorylation of SHC by integrin-associated TKs (Fyn, other Src-like, FAK, or FAK-associated kinases) and subsequent activation of MAPK [18-21]. The biological significance of integrin-stimulated MAPK activity is not well understood, however, recent data indicated that it positively regulates cell growth and survival [18, 22], but is not essential for cell migration [23].

This work addressed the consequences of amplified SHC signaling on proliferation, transformation, adhesion, and motility in breast cancer MCF-7 cells. In these cells, SHC is an important intermediate of different signaling pathways. Growth factors present in serum, such as IGF-I or EGF can induce SHC through their cognate receptors [1, 16, 24]. Estrogens (also contained in serum) may elevate tyrosine phosphorylation of SHC via cytoplasmic TKs of the Src family [25]. In addition, SHC can be stimulated by cytoplasmic TKs as a result of cell spreading on ECM [18]. MCF-7 cells express several integrin receptors: $\alpha2\beta1$, $\alpha3\beta1$, $\alpha5\beta1$, $\alpha v\beta5$ [26]. Of those, $\alpha5\beta1$, a FN receptor, is known to associate with SHC, while $\alpha2\beta1$ and $\alpha3\beta1$ are not SHC binding proteins [18].

The interactions of cells with FN have been reported to influence or control different processes regulating the behavior of cancer cells, namely cell migration, invasion, and metastasis as well as survival and proliferation [27]. The exact role of $\alpha5\beta1$ FN

receptors in tumor progression is not clear. It has been shown that extracellular matrix recognition by α5β1 integrin is a negative regulator of cell growth and may be lost in some tumor cells [28]. In agreement with this, overexpression of α5β1 integrin and improved cell spreading on FN can reduce cell growth and transformation in vivo and reverse tumorigenicity in vitro [29, 30]. On the other hand, FN receptors may play a role in later stages of tumor progression since blocking α5β1 integrin abrogated cell spread in experimental breast metastasis [31]. The importance of SHC signaling in the interactions of breast cancer cells with FN has not been studied and is a subject of this work.

MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions. MCF-7 cells are estrogen receptor positive cells of a low tumorigenic and metastatic potential. The growth of MCF-7 cells is controlled by estrogens, such as estradiol (E2), and growth factors, such as IGF-I and EGF [32, 33]. MCF-7 cells express several integrins, including α5β1 (FN receptor), α2β1 (collagen, COL receptor), α3β1 (COL/FN/laminin 5 receptor) and αvβ5 (vitronectin receptor) [26].

MCF-7/SHC clones 1 and 9 are MCF-derived cells stably transfected with the expression plasmid pcDNA3/SHC containing a human SHC cDNA encoding $p55^{SHC}$ and $p47^{SHC}$. The clones expressing the transgene were selected in 2 mg/ml G418, and the levels of SHC expression in 20 G418-resistant clones were determined by Western blotting (WB) in whole cell protein lysates, as described below.

As control cells, we used several MCF-7-derived clones with modified growth factor signaling pathways, specifically, MCF-7/IRS-1, clones 3 and 18 that are MCF-7 cells overexpressing insulin receptor substrate 1 (IRS-1) [32]; MCF-7/IGF-IR, clone 17 that is an MCF-7-derived clone overexpressing the insulin-like growth factor 1 receptor

(IGF-IR) [33], and MCF-7/anti-SHC, clone 2 with SHC levels decreased by 50% due to the stable expression of an anti-SHC RNA [16].

MCF-7 cells were grown in DMEM:F12 (1:1) containing 5% calf serum (CS). MCF-7-derived clones were maintained in DMEM:F12 plus 5% CS plus 200 ug/ml G418. In the experiments requiring E2- and serum-free conditions, the cells were cultured in phenol red-free DMEM containing 0.5 mg/ml BSA, 1 uM FeSO4 and 2 mM L-glutamine (referred to as PRF-SFM).

Monolayer Growth. Cells were plated at a concentration 1.5-2.0x10^5 in 6-well plates in the growth medium; the following day (day 0), the cells at approximately 50% confluence were shifted to PRF-SFM containing 1 or 20 ng/ml IGF-I or 1 or 10 ng/ml EGF. After 4 days, the number of cells was determined by direct counting.

Anchorage-Independent Growth. Transforming potential of the cells (anchorage-independence) was measured by their ability to form colonies in soft agar, as described before [32] The cells, 1x10^3/35 mm plate, were grown in a medium solidified with 0.2% agarose. The solidified medium contained either (i) DMEM:F12 supplemented with 10% FBS or 5% CS, or (ii) PRF-SFM with either 200 ng/ml IGF-I, 50 ng/ml EGF, or 200 ng/ml IGF-I plus 50 ng/ml EGF. After 21 days of culture, the colonies greater then 100 um in diameter were counted using an inverted phase-contrast microscope.

Adhesion on FN or COL. Cells synchronized for 24 h in PRF-SFM were seeded in 60 mm plates coated with FN (50 ug/ml) or COL (20 ug/ml). Before the experiment, the plates were blocked with 3% BSA for 3h at 37°C and then washed once with PBS. To inactivate $\alpha5\beta1$ integrin, the cells were incubated with a blocking $\alpha5\beta1$ Ab 10 ug/ml (Chemicon) for 30 min before plating. Cell morphology was recorded using an inverted phase-contrast

microscope with a camera. Percentage of non-adherent cells was determined by counting the number of floating cells vs. the number of cells originally inoculated in the plate.

Growth on FN. Cells ($0.5x10^5$/ml) were seeded in 12-well plates coated with FN (50 ug/ml) in normal growth medium with or without EGF (10, 50 or 100 ng/ml) or IGF-I (20 or 100 ng/ml). The cells were counted after 4 days of culture.

Motility Assay. Motility was tested in modified Boyden chambers containing porous (8 mm) polycarbonate membranes. The underside of membranes was coated with either 20 ug/ml COL IV or 50 ug/ml FN, as described by Mainiero et al [34]. According to this protocol, COL or FN cover not only the underside of the membrane, but also diffuse into the pores where cell contact with ECM is initiated. $2x10^4$ of synchronized cells suspended in 200 ul of PRF-SFM were plated into upper chambers. Lower chambers contained 500 ul of PRF-SFM with EGF (1 and 10 ng/ml) or IGF-I (20 ng/ml). After 12 h, the cells in the upper chamber were removed, while the cells that migrated to the lower chamber were fixed and stained in Coomassie Blue solution (0.25g Coomassie blue/45 ml water/45ml methanol/10 ml glacial acetic acid) for 5 min. After that, the chambers were washed 3 times with H_2O. The cells that migrated to the lower chamber were counted under the microscope as described before [16].

Immunoprecipitation and Western Blotting. Proteins were obtained by lysis of cells with a buffer containing: 50 mM HEPES pH 7.5, 150 mM NaCl, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 100 mM NaF, 0.2 mM Na_3VO_4, 1% PMSF, 1% aprotinin. The expression of SHC in transfectants and the parental cells was assessed in 50 ug of total cell lysate using an anti-SHC monoclonal antibody (mAb) (Transduction Laboratories). Alternatively, SHC was detected by immunoprecipitation (IP) from 250-1000 ug (specific amounts are given under the figures) of protein lysate with an anti-SHC polyclonal

antibody (pAb) (Transduction Laboratories), followed by WB with an anti-SHC mAb (Transduction Laboratories). Tyrosine phosphorylation of SHC was measured by WB using an anti-phosphotyrosine mAb PY20 (Transduction Laboratories). The levels of $\alpha5\beta1$ integrin were assessed in 1 mg of protein lysate by IP with an anti-$\alpha5\beta1$ pAb (Chemicon) and WB using an anti-$\beta1$ mAb (Chemicon). The amounts of integrin-associated SHC were measured in $\alpha5\beta1$ integrin immunoprecipitates with an anti-SHC pAb (Chemicon). The intensity of bands representing relevant proteins was measured by laser densitometry scanning.

MAPK Activity. The phosphorylated forms of p42 and p44 MAPK were identified by WB in 50 ug of whole cell lysates with an anti-phospho-MAPK (Thr202/Tyr204) mAb (New England Biolabs). The total levels of MAPK were determined with an anti-MAPK pAb (New England Biolabs). Adhesion-induced MAPK activity was assessed in cells plated either on COL IV or FN and then lysed at different times after plating (0-24h). To determine EGF-induced MAPK activity, the cells were plated on different substrates, allowed to attach for 1h, and then treated with 10 ng/ml EGF. The cells were lysed at different times (0-24h) of the treatment and MAPK activity was measured as described above.

Statistical Analysis. The results of cell growth experiments were analyzed by Student t-test.

RESULTS

Basal and growth factor-induced SHC tyrosine phosphorylation is increased in MCF-7/SHC cells. To investigate the implications of increased SHC signaling in breast cancer cells, we developed several MCF-7-derived clones stably overexpressing $p46^{SHC}$ and $p52^{SHC}$. Out of 20 G418 resistant clones, 7 exhibited SHC overexpression, as determined by WB (data not shown). Two representative clones, MCF-7/SHC, 1 and MCF-7/SHC, 9, with a 7- and 9-fold SHC amplification, respectively, were selected for subsequent experiments (Fig. 1). The greater amount of SHC in these clones was reflected by increased levels of SHC tyrosine phosphorylation, which was evident in both continuously proliferating (Fig. 1A) and EGF-stimulated cultures (Fig. 1B). The extent of SHC tyrosine phosphorylation roughly corresponded to the cellular levels of the protein (Fig. 1A and B).

Overexpression of SHC has minimal effects on cell growth on plastic and does not enhance transformation in soft agar. The significant hyperactivation of SHC in MCF-7/SHC cells suggested that growth properties of these cells might have been altered. First we determined that in serum-containing medium or PRF-SFM, the growth rate of MCF-7/SHC clones was comparable to that of the parental cells or other cell lines with normal SHC levels (data not shown). Next, we studied mitogenic response to EGF and IGF-I, growth factors which stimulate tyrosine phosphorylation of SHC [1, 24] and require SHC for their growth action [16]. We found that relative to MCF-7 cells, MCF-7/SHC clones exhibited only moderately (20-40%) enhanced growth response to IGF-I or EGF (Fig. 1C). Under the same conditions, the proliferation of a control clone MCF-7/anti-SHC, 2 was substantially (at least by 50%) reduced (Fig. 1C), as demonstrated by us before [16].

Since SHC is oncogenic when overexpressed in NIH mouse fibroblasts [1], we assessed transforming potential of MCF-7/SHC clones in soft agar assay. Despite significant SHC overexpression in these cells, in several repeat experiments and under

different growth conditions used, anchorage-independent growth of MCF-7/SHC cells was never enhanced relative to that seen in MCF-7 cells (Tab. 1). In the same assay, MCF-7-cells overexpressing IRS-1, i.e., MCF-7/IRS-1, clone 3, exhibited typical for these cells, increased transformation in the presence of serum or IGF-I [32].

SHC associates with α5β1 integrin (FN receptor) in MCF-7 cells. The abundance of SHC/α5β1 integrin complexes is increased in MCF-7/SHC cells. The limited or absent effects of SHC overexpression on mitogenic and transforming potential of MCF-7 cells prompted us to assess the role of SHC in non-growth processes, specifically, adhesion and motility. Because interactions of cells with FN have been implicated in the growth and metastatic behavior of breast cancer cells [30, 31] and since SHC is a potential mediator of FN receptor signaling [18, 21], we investigated how overexpressed SHC affects the function of α5β1 integrin in MCF-7 cells. The first observation was that the levels of SHC, especially $p46^{SHC}$, associated with α5β1 were markedly increased (~ 7-fold) in MCF-7/SHC clones compared with that in MCF-7 cells or in several cell lines with normal levels of SHC but overexpressing other signaling proteins (IRS-1 or the IGF-IR) (Fig. 2). Interestingly, the amount of $p46^{SHC}$ associated with α5β1 integrin was similar in both MCF-7/SHC clones, regardless of the level of SHC overexpression (Fig. 2). This suggests that the extent of SHC/α5β1 binding is not directly proportional to total cellular SHC levels and that α5β1/SHC complex formation is a saturable process, possibly determined by the limited expression of α5β1 integrin in MCF-7 cells [30].

MCF-7/SHC cells exhibit increased adhesion to FN. Because of the enhanced association of SHC with α5β1 integrin in MCF-7/SHC cells, we examined the role of SHC in cellular interactions with FN using cell lines with normal, amplified, or reduced SHC levels. We found that the overexpression of SHC was associated with an accelerated cell adhesion to FN, while the reduction of SHC levels blocked cell spreading on the substrate (Fig. 3 and Tab. 2). The differences in the dynamics of cell interactions with FN were most evident at 1 h after plating (Fig. 3, panels B and Tab. 2). Specifically, while at this time both MCF-7/SHC clones were well spread on FN, and almost no floating cells were observed, only ~50% of MCF-7 cells exhibited initial attachment to the substrate (cells were still rounded but with distinct membrane protrusions), and MCF-7/anti-SHC cells remained completely suspended. At 2 and 6h after plating, MCF-7 and MCF-7/SHC clones were attached to FN and the differences in adhesion among these cell lines were unremarkable. At the same time, MCF-7/anti-SHC cells were in minimal contact with FN (Tab. 2). After 24 h, MCF-7/anti-SHC cells formed small aggregates demonstrating limited contact with the substrate, but all other tested cell lines (represented here by MCF-7 cells) were fully attached (Fig. 3, panels D and Tab. 2). At 48 h, MCF-7/anti-SHC cells were completely detached, while MCF-7 and MCF-7/SHC cells begun proliferation on FN (data not shown).

Our experiments also indicated that the adhesion of MCF-7/SHC cells to FN was mediated by α5β1 integrin, since this process was totally blocked with a specific anti-α5β1 blocking antibody (Fig. 3, panels C), but not with a control goat IgG (not shown).

In contrast with the results obtained on FN, the dynamics of cell adhesion on collagen (COL), which is mediated by an integrin not associating with SHC (α2β1) [18], was similar in all tested cell lines, regardless of the levels of SHC expression. Specifically, all cells tested initiated contacts with COL at 15 min and completed attachment at 1 h after plating (data not shown).

Overexpression of SHC modulates adhesion-dependent, but not growth factor-induced, MAP kinase activity on FN. Cell adhesion to ECM and the activation of different integrin-associated cytoplasmic TKs results in the stimulation of MAPK activity [35]. This process can be mediated through SHC, which, as a substrate of TKs (e.g., Fyn, other c-Src-like kinases, or FAK), is tyrosine phosphorylated, binds the GRB2/SOS complex and stimulates Ras [18, 19, 21]. The integrin-MAPK pathway can also be induced in a SHC-independent way, through FAK-GRB 2-SOS-Ras signaling [20, 21]. Here we studied the effect of SHC amplification on adhesion-dependent MAPK response in MCF-7 and MCF-7/SHC cells. Fig 4 demonstrates representative results obtained with MCF-7/SHC, 1 cells; the results with the clone MCF-7/SHC, 9 were similar.

First, we found that overexpression of SHC significantly modulated MAPK activation in cells spread on FN, but on COL (Fig. 4 A). Specifically, on COL, MCF-7 and MCF-7/SHC cells responded similarly--the activation of MAPK was bi-phasic, with a peak between 30 min and 4 h after plating, followed by a decline of activity at 8 h, and then an increased activity between 12 and 24 h. In contrast, on FN, the dynamics of MAPK response was different--in MCF-7 cells, the stimulation of MAPK was the highest at 1h after plating and the kinases remained highly stimulated for up to 8 h. In MCF-7/SHC cells, MAPK were activated at 30 min after plating, reached the maximum at 1 h, and rapidly declined at 4 h to reach basal levels at 24 h (Fig. 4 A). The activation of MAPK in suspended cells was undetectable (not shown).

Next, we investigated whether SHC overexpression affects growth factor-induced MAPK response in cells plated on FN. We used EGF in this experiment since this mitogen stronger induces SHC phosphorylation than IGF-I in MCF-7 cells (Surmacz et al., unpublished observations). The pattern of EGF-stimulated MAPK activity was remarkably

similar on COL and FN (a peak between 15 min and 1 h after treatment followed by a decline to basal levels) in both MCF-7 and MCF-7/SHC cells (Fig. 4B).

Since MAPK pathway contributes to cell growth, we studied whether the reduced duration of adhesion-dependent MAP activity reflects mitogenicity of MCF-7/SHC cells cultured on FN (Tab. 3). Indeed, we found that overexpression of SHC coincided with a significant (~2-fold) growth inhibition. Interestingly, the addition of EGF (different doses, up to 100 ng/ml) to growth medium did not improve proliferation of MCF-7/SHC or MCF-7 cells on FN. The addition of IGF-I (doses up to 100 ng/ml) only minimally (9-22%) stimulated growth under the same conditions (Tab. 3).

Overexpression of SHC inhibits basal motility on FN. IGF-I or EGF mobilize MCF-7/SHC cells. We investigated whether increased binding of SHC to $\alpha 5\beta 1$ integrin affects cell motility in FN-coated inserts. We found that basal migration of MCF-7/SHC cells was significantly (~4-fold) reduced compared with that of MCF-7 cells and several MCF-7-derived cell lines containing normal amounts of SHC (Fig. 5). In contrast, the motility of MCF-7/SHC clones in COL-coated inserts was similar ($p \geq 0.05$) to that seen with other tested cell lines (Fig. 5).

The use of EGF or IGF-I as chemoattractants significantly (~5-7-fold, $p \leq 0.01$) improved the migration of MCF-7/SHC cells towards FN, but not to COL. The mitogens did not affect motility of other cells to COL, except some inhibition of MCF-7/IRS-1, clone 18 with 10 ng/ml EGF. Interestingly, in FN-coated inserts, 10 ng/m EGF stimulated the migration of MCF-7/IRS-1 cells, however, the extent of this stimulation was much lower than that of SHC overexpressing clones (Fig. 5). The increased EGF sensitivity of MCF-7/IRS-1 clones has been noticed before [16].

DISCUSSION

SHC is a signaling substrate of most receptor-type and cytoplasmic TKs and therefore may amplify various cellular responses [2]. In consequence, the significance of SHC amplification must depend on the intracellular and extracellular cell context. Breast cancer cells, unlike normal breast epithelium, frequently overexpress TKs, such as c-Src (80%), or ERB2 (~20-30%), which may result in constitutive activation of SHC [9, 10]. The contribution of amplified SHC signaling to development and progression of breast cancer is not known. We addressed this question by examining the effects of SHC overexpression in MCF-7 cells (representing an early stage of breast cancer and characterized by moderate c-Src amplification). The major findings of this work are that unlike in fibroblasts, hyperactivation of SHC is not sufficient to provide significant growth or transforming advantage in breast cancer cells. High levels of SHC, however, increase cell connections with FN and modulate cell growth and migration on this substrate, which may have consequences in cell spread and metastasis.

SHC in epithelial cell growth and transformation. In mouse fibroblasts, overexpression of SHC resulted in increased SHC tyrosine phosphorylation, augmented EGF-, or IGF-I-dependent MAPK response, accelerated cell cycle progression through G1 phase in the absence of growth factors, and enhanced anchorage-independent growth in soft agar and tumorigenicity in nude mice [1, 6, 24]. Increased levels of SHC also potentiated growth factor response in myeloid and A549 adenocarcinama cells [3,5]. Consistent with these findings are our previous data demonstrating that downregulation of SHC results in reduced sensitivity to mitogenic action of EGF and IGF-I and growth inhibition in breast cancer cells [16]. The present studies indicated that in SHC overexpressing epithelial cells, like in fibroblasts, basal and growth factor-induced SHC tyrosine phosphorylation was increased, and cell responsiveness to IGF-I and EGF was moderately augmented in monolayer culture on plastic. However, amplification of SHC did not potentiate MAPK activity or proliferation of cells in complete serum-containing

medium. This, again, was consistent with the effects observed in SHC overexpressing NIH 3T3 fibroblasts [6].

Noteworthy, high levels of SHC did not promote transformation of MCF-7 cells, whereas overexpression (at a similar level) of another signaling substrate IRS-1 markedly augmented anchorage-independent growth [32]. Since anchorage-independent growth reflects tumorigenic potential of breast cancer cells [36] and other cell types [1], our results indicate that, unlike in NIH 3T3 cells, SHC is not oncogenic in MCF-7 cells. This may reflect differences between pathways controlling transformation in fibroblasts and epithelial cells.

SHC in cell adhesion and motility. In contrast with the little impact of SHC overexpression on growth and transforming processes, high levels of SHC significantly modulated cell interactions with ECM in breast epithelial cells. SHC was found associated with $\alpha5\beta1$ integrin, the FN receptor, and $\alpha5\beta1$/SHC complexes were more abundant in SHC overexpressing cells than in other cell lines with SHC normal levels. The increased SHC/$\alpha5\beta1$ binding in MCF-7/SHC cells was paralleled by accelerated cell attachment to FN, reduced basal motility, abbreviated adhesion-mediated MAPK activity, and inhibited proliferation on the substrate. These effects were absent on COL (whose receptor does not bind SHC in MCF-7 cells), which suggests a specific role of SHC-$\alpha5\beta1$ interactions in the above processes.

The association of SHC with certain classes of integrins has been noted in several other cell systems. In A431 cells and other cell lines, binding and tyrosine phosphorylation of SHC to $\beta1$ integrin was induced by cell contact with ECM or by integrin cross-linking with a specific antibody [18]. Similarly, an association of SHC with $\alpha6\beta4$ integrin was observed in attached, but not suspended, A431 cells [34]. In several cell types, ligation of SHC-binding integrins, but not other integrins, has been reported to enhance cell cycle

progression [18]. In our cell system, however, the increased association of SHC with α5β1 integrin and the enhanced attachment to FN, coincided with growth inhibition. Consistent with these findings are the observations of Wang et al., who reported that in MCF-7 cells, α5β1 integrin overexpression and improved interactions of cells with FN resulted in reduced proliferation on the substrate and impaired tumorigenicity in vivo [30].

Cell growth and survival on ECM is reflected by enhanced MAPK activity [18, 19, 35]. This pathway is induced by various integrin-associates TKs (e.g., c-Src-like TKs or FAK) and often involves activation of the SHC-GRB2-Ras pathway [19-21]. We found that the amplification of SHC corresponded to the reduced duration of adhesion-mediated MAPK response on FN but not on COL. Noteworthy, in mouse fibroblasts, an early decline of MAPK activity coincided with growth inhibition, whereas a prolonged activity marked cell cycle progression [37]. Thus, the abbreviated MAPK response in MCF-7/SHC cells may reflect their significantly slower proliferation on FN. In our experiments, treatment of cells spread on FN with EGF induced MAPK but did not stimulate cell growth, which confirms that MAPK signaling is required but not sufficient for the proliferation of MCF-7 cells [38].

Reduced growth and better attachment to FN in MCF-7/SHC cells was also associated with significantly reduced basal migration. However, EGF or IGF-I stronger induced motility of MCF-7/SHC clones than other control cell lines when tested in FN-coated inserts. Such an enhancement of growth factor-induced migration in SHC overexpressing cell lines has been reported before [5]. The increased chemotaxis was probably mediated by pathways other than MAPK, since MAPK activity was similar in MCF-7 and MCF-7/SHC cells treated with EGF.

In summary, in MCF-7 cells, the impact of the amplified SHC on cell growth and transformation is not significant, however, SHC plays an important role in the regulation of cell adhesion and motility on FN through its interaction with α5β1 integrin. The

significance of SHC-mediated interactions with FN in breast cancer metastasis are not known and will be pursued in an animal model.

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TABLES

Tab. 1. Anchorage-Independent Growth of MCF-7/SHC Cells

Number of Colonies					
Cell Line	10% FBS	5% CS	SFM+ IGF-I	SFM+ EGF	SFM+ IGF-I +EGF
MCF-7	172	105	2	1	12
MCF-7/SHC, 1	164	90	0	0	9
MCF-7/SHC, 9	155	88	0	0	10
MCF-7/IRS-1, 3	213	131	25	10	22

The cells were tested in soft agar as described under Materials and Methods. The agar-solidified medium was either DMEM:F12 with 10% FBS, or 5% CS, or PRF-SFM with EGF (200 ng/ml), IGF-I (50 ng/ml), or EGF plus IGF-I (50 ng/ml plus 200 ng/ml, respectively). MCF-7/IRS-1, clone 3, characterized by an increased transforming potential [30], was used as a positive control. The experiment was repeated 7 times. Average number of colonies of the size at least 100 um in diameter is given.

Tab. 2. Dynamics of cell attachment to FN

	% Non-adherent Cells					
Cell Line	0h	0.5h	1h	2h	6h	24h
MCF-7	100	74	55	8	5	2
MCF-7/SHC, 1	100	33	5	5	4	4
MCF-7/SHC, 9	100	25	6	5	2	3
MCF-7/anti-SHC, 2	100	99	100	78	80	76

$0.5x10^5$ cells suspended in PRF-SFM were plated into 60 mm plates coated with 50 ug/ml FN as described under Materials and Methods. At the time of plating (0h) and at 0.5, 1, 2, 6, and 24h after plating, the floating cells were collected and counted. The values represent % cells floating vs. cells originally plated, and are average from 3 experiments.

Tab. 3. Growth of MCF-7/SHC cells on FN

	Cell Number		
Cell Line	5%CS	5% CS +EGF	5% CS+ IGF-I
MCF-7	$2.2x10^5$	$2.2x10^5$	$2.4x10^5$
MCF-7/SHC, 1	$1.1x10^5$	$1.0x10^5$	1.3x10
MCF-7/SHC, 9	$0.9x10^5$	$0.8x10^5$	$1.1x10^5$

The growth of cells on FN in either normal growth medium (DMEM:F12 + 5% CS) or growth medium containing EGF (100 ng/ml) or IGF-I (100 ng/ml) was tested as described under Materials and Methods. The cells were plated at the concentration $0.5x10^5$ cells/ml and counted after 4 days of culture. The values represent cell number/ml, and are average results from 3 independent experiments.

FIGURE LEGENDS

Fig. 1. MCF-7/SHC cells. A. SHC expression and tyrosine phosphorylation in proliferating cells. The protein levels and tyrosine phosphorylation (PY) of $p52^{SHC}$ and $p46^{SHC}$ in two selected MCF-7/SHC clones 1 and 9 were determined in 750 ug of protein lysate by IP and WB with specific antibodies, as detailed under Materials and Methods. Cell lysates were isolated from logarithmic cultures maintained in normal growth medium. **B. SHC expression and tyrosine phosphorylation in growth factor-stimulated cells.** 70% confluent cultures were synchronized in PRF-SFM for 24 h and then stimulated with 10 ng EGF for 15 min. SHC levels and tyrosine phosphorylation (PY) were studied by IP and WB in 250 ug of protein lysates. Note that lane MCF-7/SHC, 1, EGF (-) is overloaded. **C. Growth response of MCF-7/SHC cells to IGF-I and EGF.** The cells at 50% confluence were synchronized in PRF-SFM and stimulated with mitogens for 4 days as described in Materials and Methods. Abscissa, cell lines; ordinate, the percentage of growth increase over that in PRF-SFM. Dotted bars, low doses: IGF-I 1ng/ml or EGF 1 ng/ml; striped bars, high doses, IGF-I 20 ng/ml or EGF 10 ng/ml. High doses of IGF-I or EGF are the EC_{50} concentrations in these cells. SD is marked by solid bars; asterisks indicate statistically significant differences ($p \leq 0.05$) between the growth responses of MCF-7/SHC or MCF-7/anti-SHC cells and identically treated MCF-7 cells. The results are average of 4 experiments.

Fig. 2. SHC associates with $\alpha 5\beta 1$ integrin. The amounts of SHC associated with $\alpha 5\beta 1$ integrin in MCF-7/SHC cells, MCF-7 cells, and several control clones with normal SHC levels but increased levels of IRS-1 (MCF-7/IRS-1, clones 3 and 18) or the IGF-IR (MCF-7/IGF-IR, clone 17) were determined in 750 ug of protein lysate by IP with an anti-

α5β1 pAb and WB with an anti-SHC pAb (**A**). The expression of α5β1 integrin in the cells was determined after stripping the above blot and re-probing with the anti-β1 pAb (only the β subunit is shown) (**B**). To locate the position of SHC isoforms on the gel, SHC proteins were precipitated from 250 ug of MCF-7 and MCF-7/SHC, 1 cell lysates with an anti-SHC pAb, run in parallel with α5β1 integrin IP samples and probed with an anti-SHC mAb (**C**). Note: The α5β1 integrin IP samples could not be re-probed with the SHC mAb because of strong antibody cross-reaction.

Fig. 3. Adhesion of MCF-7/SHC clones on FN. MCF-7/SHC clones 1 and 9 (amplified SHC), MCF-7 cells (normal SHC levels) and MCF-7/anti-SHC, clone 2 (reduced SHC levels) were synchronized for 24h in PRF-SFM and plated on FN (50 ug/ml) in PRF-SFM. The cells were photographed at times 0 (**A**), and 1h (**B**). The role of α5β1 integrin in the adhesion of MCF-7/SHC clones 1 and 9 was assessed by blocking the FN receptor with a specific antibody 30 min before cell plating (**C**), as described in Materials and Methods. The long-term (24 h) adhesion of MCF-7 and MCF-7/anti-SHC, clone 2 is shown in panels **D.**

Fig. 4. Adhesion-induced (A) and EGF-dependent (B) MAPK activity in MCF-7/SHC cells. To measure adhesion-induced MAP kinase activity (**A**), MCF-7 and MCF-7/SHC cells were plated on COL IV or FN. The cells were lysed at the indicated times after plating. The phosphorylated forms of p42 and p44 MAPK were determined as described under Materials and Methods. EGF-induced MAP kinase activity (**B**) was determined in MCF-7 and MCF-7/SHC cells. The cells were plated on COL IV or FN, allowed to attach for 1h, and then treated with 10 ng/ml EGF. The cells were lysed at different times (0-24h) of the treatment. In (**A**) and (**B**), panels (**a**) represent

phosphorylated MAPK, panels (**b**) total cellular levels of MAPK. The representative results demonstrating MAPK response in MCF-7 cells and MCF-7/SHC, clone 1 are shown; results with MCF-7/SHC, clone 9 were analogous to that obtained in clone 1.

Fig. 5. Motility of MCF-7/SHC cells in FN or COL inserts. The motility of MCF-7/SHC cells and several control cell lines with normal SHC levels was tested as described under Materials and Methods. The upper and lower chambers contained PRF-SFM. Growth factor-induced motility was assessed by supplementing PRF-SFM in lower chambers with either EGF (1 or 10 ng/ml) or IGF-I (20 ng/ml). The percentage of cells that migrated to the underside of inserts (relative to the number of cells plated) is designated as % Motility. The experiments were repeated 4 times. Average values are given. Asterisks indicate statistically significant (*, $p \leq 0.05$, **, $p \leq 0.01$) differences between the basal and growth factor induced migration of a given cell line.

Figure 1a

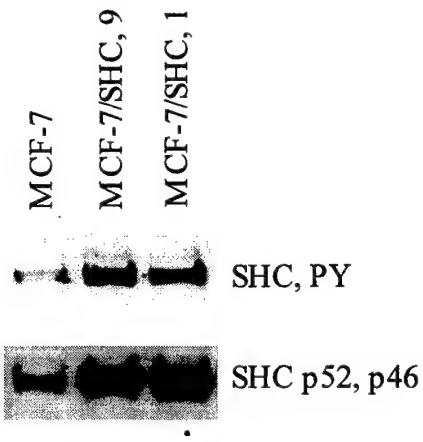


Figure 1b

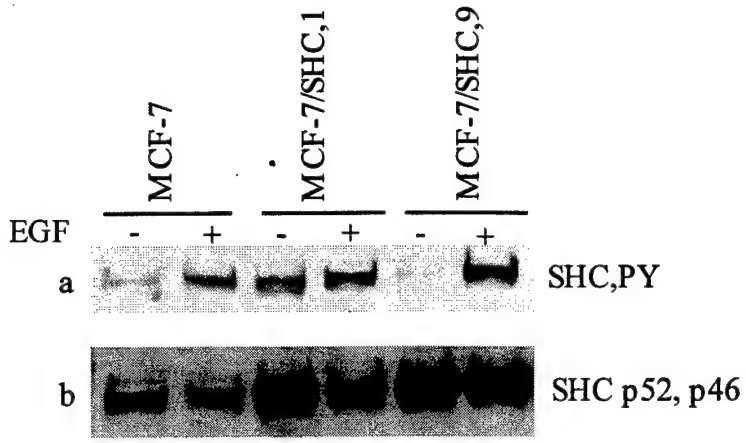


Figure 1C

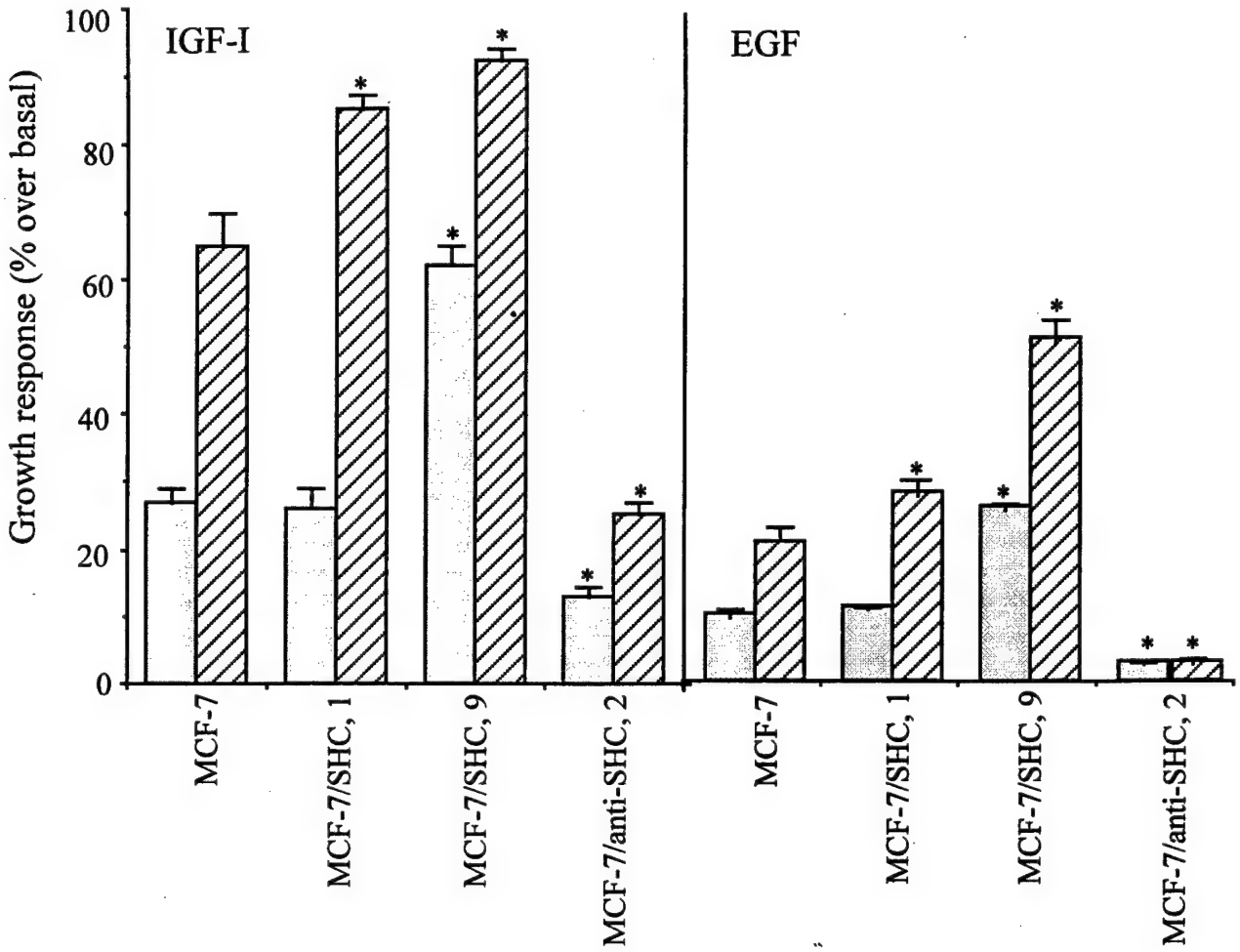

IGF-I
EGF
Growth response (% over basal)
100
80
60
40
20
0
MCF-7
MCF-7/SHC, 1
MCF-7/SHC, 9
MCF-7/anti-SHC, 2
MCF-7
MCF-7/SHC, 1
MCF-7/SHC, 9
MCF-7/anti-SHC, 2

Figure 2

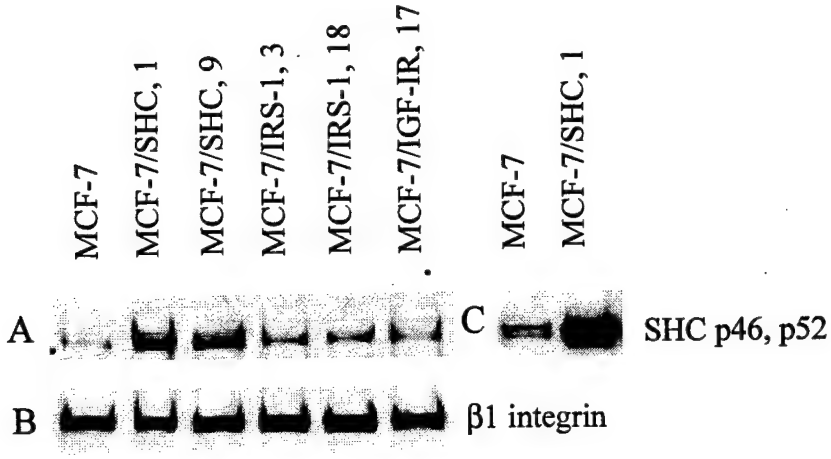

MCF-7
MCF-7/SHC, 1
MCF-7/SHC, 9
MCF-7/IRS-1, 3
MCF-7/IRS-1, 18
MCF-7/IGF-IR, 17
MCF-7
MCF-7/SHC, 1
A
C
SHC p46, p52
B
β1 integrin

Figure 3

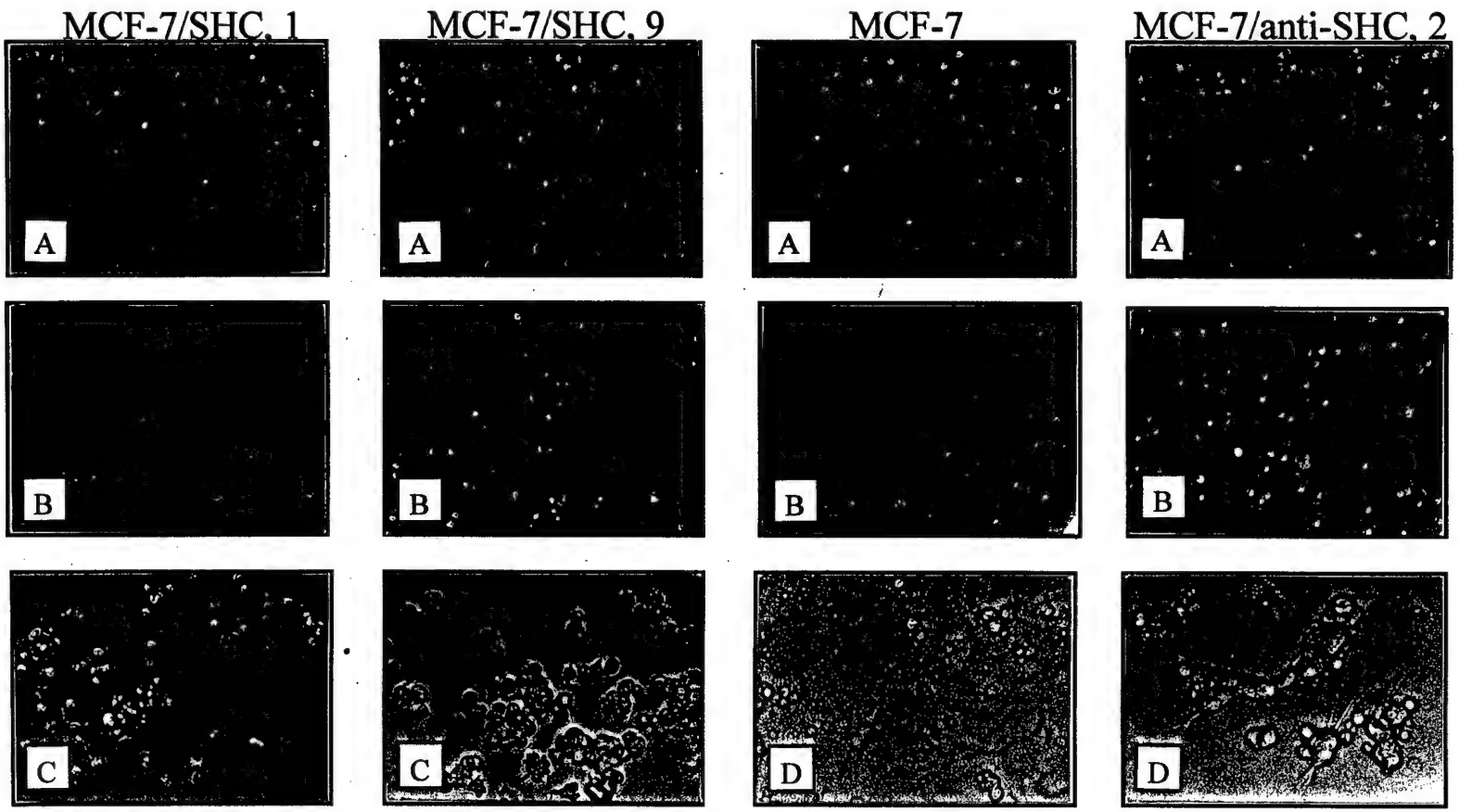
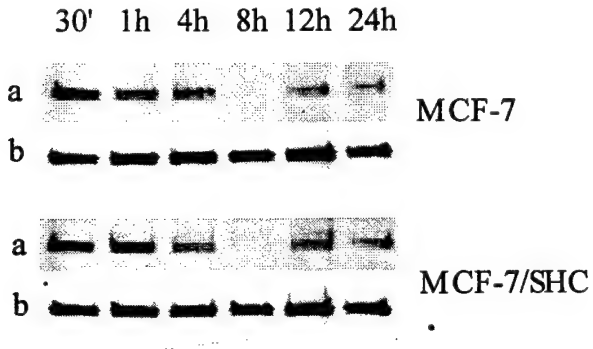


Figure 4a

Collagen



Fibronectin

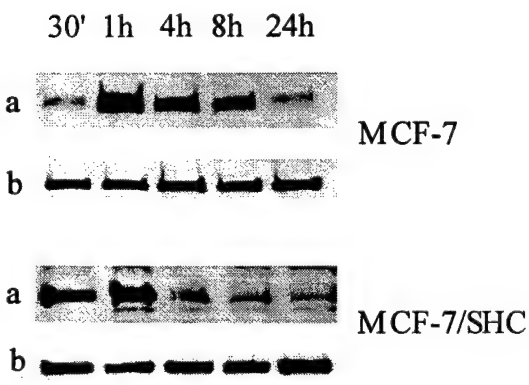


Figure 4b

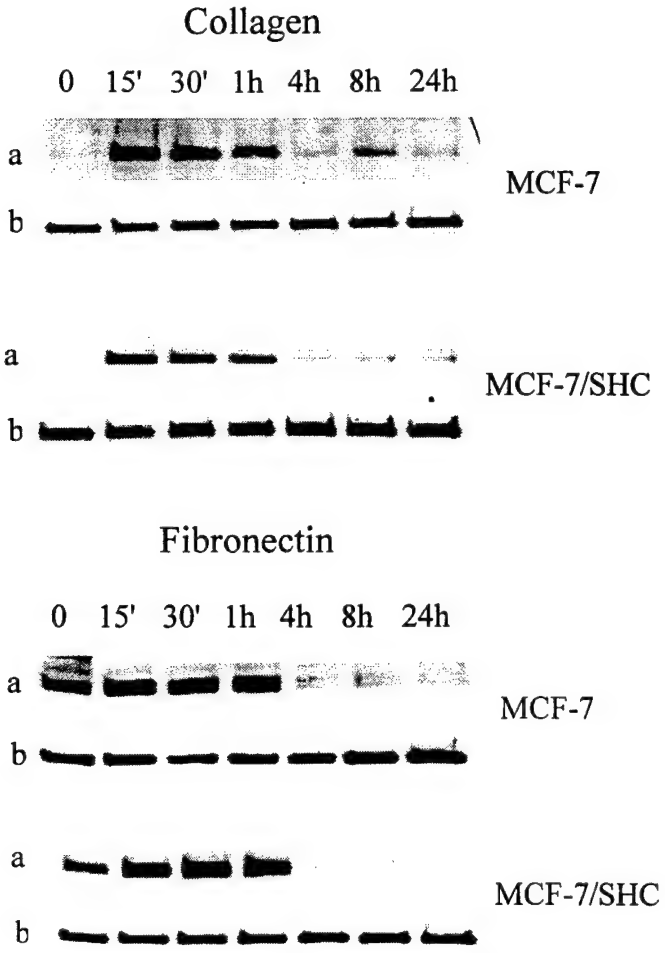
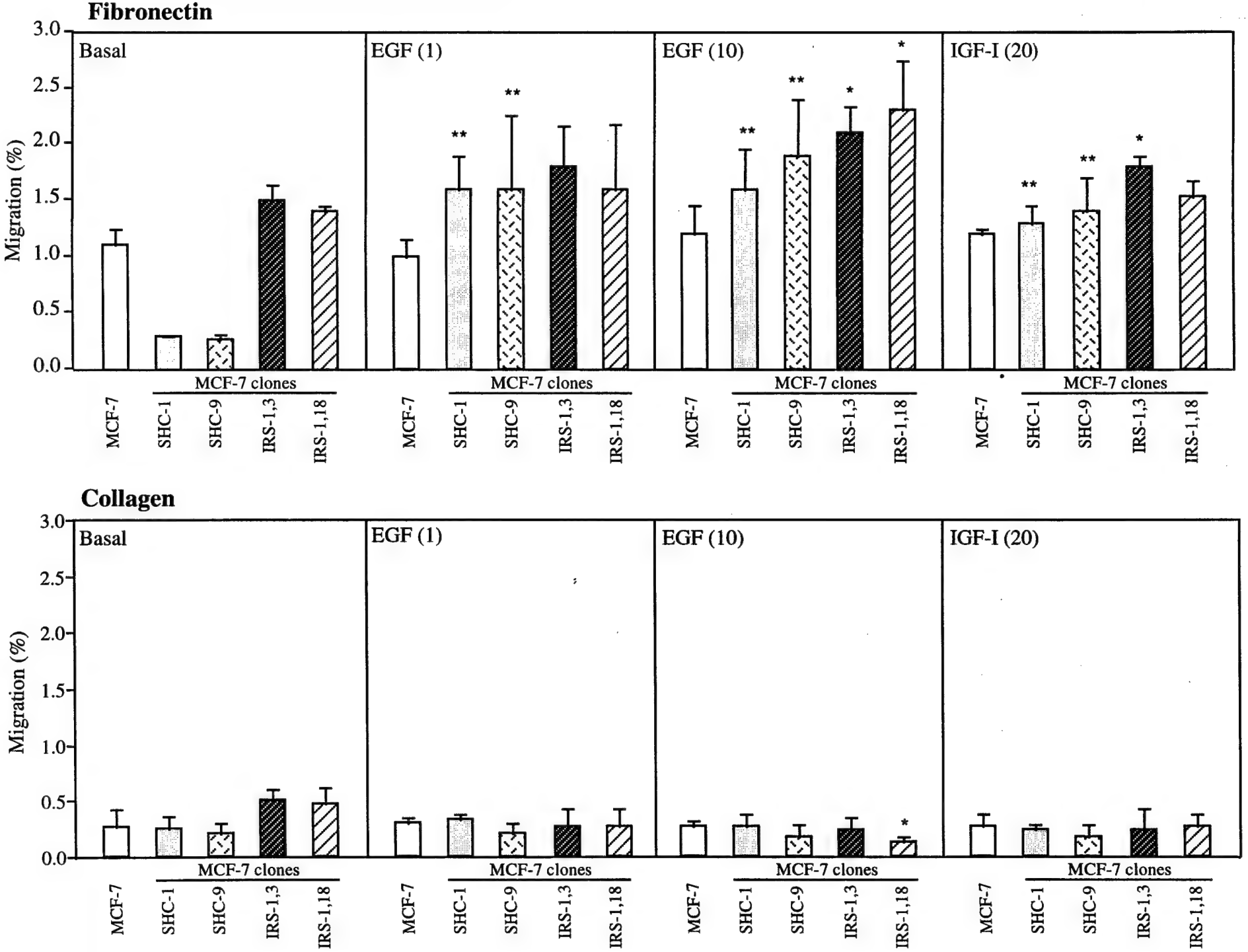

Collagen
0 15' 30' 1h 4h 8h 24h
a
b
MCF-7
a
b
MCF-7/SHC
Fibronectin
0 15' 30' 1h 4h 8h 24h
a
b
MCF-7
a
b
MCF-7/SHC

Figure 5


Fibronectin
Basal
EGF (1)
EGF (10)
IGF-I (20)
Migration (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MCF-7 clones
MCF-7
SHC-1
SHC-9
IRS-1,3
IRS-1,18
Collagen
Basal
EGF (1)
EGF (10)
IGF-I (20)
Migration (%)
MCF-7 clones
MCF-7
SHC-1
SHC-9
IRS-1,3
IRS-1,18

IGF-I Receptors Stimulate Motility in Breast Cancer Epithelilal Cells through Reorgnization of the Actin Cytoskeleton and Focal Contacts, and Modulation of Phosphorylation Status of FAK, Cas and Paxillin.

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Running tittle: IGF-IR-mediated motility in breast cancer cells

Key words: IGF-IR signaling, F-actin, Focal adhesion, PTPase activity

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SUMMARY

Insulin-like growth factor I (IGF-I) is known to promote the motility of different cell types. We investigated the role of IGF-I receptor (IGF-IR) signaling in motility of MCF-7 breast epithelial cells overexpressing the wild type IGF-IR. The elevated level of the IGF-IR correlated with augmented migration of cells in vitro. The treatment of subconfluent, serum-starved cells with 50 ng/ml IGF-I induced rapid morphological transition towards mesenchymal phenotype and resulted in disruption of polarized cell monolayer. Immunofluorescence staining of IGF-I-treated cells with rhodamine-phalloidin revealed dynamic changes in the actin cytoskeleton marked by disassembly of long actin fibers within 5-15 min, followed by the development of a meshwork of short actin bundles localized to multiple membrane protrusions. In parallel, IGF-I induced dephosphorylation of focal adhesion associated proteins: p125 focal adhesion kinase (FAK), p130 Crk-associated substrate (Cas) and paxillin. Pretreatment of cells with 5 μM phenylarsine oxide (PAO), an inhibitor of phosphotyrosine phosphatases, rescued FAK and its associated proteins Cas and paxillin from IGF-I-induced tyrosine dephosphorylation. PAO-pretreated cells were refractory to morphological transition and did not develop membrane protrusions in response to IGF-I. Additionally, PAO inhibited migration of these cells in a time and concentration dependent manner. Our results suggest that the activated IGF-IR stimulates a putative protein tyrosine phosphatase, which either directly or indirectly acts upon the focal adhesion proteins and promotes the reorganization of the paxillin-containing focal contacts. This process is associated with dynamic remodeling of the actin cytoskeleton. Coordinated regulating of these events is required for initiation of MCF-7 cell motility.

INTRODUCTION

The IGF-IR belongs to the tyrosine kinase receptor superfamily, and plays an important regulatory role in normal and abnormal growth (Sepp-Lorenzino, 1998; Baserga, 1998). IGF-I binding stimulates autophosphorylation of the IGF-IR beta subunit that leads to an increase in its kinase activity, and an attraction and phosphorylation of several substrates including insulin receptor substrate 1 (IRS-1). IRS-1 molecule possesses multiple tyrosine phosphorylation sites, by which this substrate recruits numerous secondary signaling proteins containing Src-homology 2 (SH2) domains including the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-kinase), phosphotyrosine phosphatase 1D (PTP1D/SH-PTP2/Syp), Src-family kinase Fyn (Yenush and White, 1997). IRS-1 also interacts with several adapter proteins Grb-2, Crk and Nck, which participate in the mitogenic Ras/Raf/MAPK (mitogen-activated protein kinase) cascade.

The accumulating data suggest that IGF-I is not solely a mitogenic factor, but it is also an important regulator of cell motility (Ando and Jensen, 1993; Leventhal and Feldman, 1997; Brooks et al., 1997; Henricks et al., 1998). In breast cancer cells, the IGF-I-induced chemotactic migration in vitro has been reported to occur through the IGF-IR, strongly suggesting the regulatory role of IGF-IR signaling in locomotion of this cell type (Kohn et al., 1990; Doerr and Jones, 1996). However, the IGF-IR signaling pathways controlling cell motility are not well defined.

The first event associated with cell movement is thought to be membrane ruffling. It is noteworthy that IGF-I has been shown to induce rapid ruffle formation in human epidermoid carcinoma KB cells, (Kadowaki et al., 1986; Miyata et al., 1989). This process involved IGF-IR autophosphorylation, binding and activation of IRS-1, and

subsequent induction of PI3-kinase (Izumi et al., 1988; Kotani et al., 1994). The precise mechanisms of ruffling are not known, though it is generally believed that polymerization of actin at the inner surface of the plasma membrane plays a crucial role (Mellström et al., 1988). Interestingly, increased concentration of actin filaments has been observed at the edges of IGF-I-stimulated epidermoid carcinoma and neuroblastoma cells (Kodowaki et al, 1986; Leventhal and Feldman, 1997). In breast epithelial cells, the relationship between IGF-IR signal transduction and actin cytoskeleton reorganization is presently unknown.

In ruffling cells, the subsequent step promoting cell motility is the development of membrane advances, followed by their outward extension and stabilization by cell-substratum adhesion (Bailly et al., 1998). The latter is accompanied by the increased tyrosine phosphorylation of the focal adhesion-associated proteins such as FAK, paxillin, tensin, cortactin, and Cas. The most extensively studied cytosolic tyrosine kinase FAK binds to a number of signaling molecules including Src family kinases, Cas, Grb2, PI3-kinase, Graf (Chen and Guan, 1994; Hanks and Polte, 1997). In attached fibroblasts, FAK is localized to focal adhesions, where it interacts with the proteins involved in the reorganization of the cytoskeleton including paxillin, talin and possibly tensin (Craig and Johnson, 1996).

In different cell types, IGF-I was shown to modulate in either a positive or negative manner the tyrosine phosphorylation of focal adhesion proteins. For example, IGF-I stimulated dephosphorylation of FAK and paxillin in CHO cells overexpressing the insulin receptor (IR) (Konstantopoulos and Clark, 1996), whereas in human neuroblastoma SH-SY5Y cells, IGF-I induced tyrosine phosphorylation of the same

adhesion proteins (Leventhal et al., 1997). Thus, the biological relevance of a bimodal effect of IGF-I on the phosphorylation status of focal adhesion proteins remained unclear. Only the recent experiments shed light on this puzzling phenomenon demonstrating that the vector of IGF-I's action at least upon FAK depends on the extent of cell-substratum adhesion rather than type of tested cells. In attached NIH 3T3 cells overexpressing the IGF-IR, IGF-I promoted tyrosine dephosphorylation of FAK, whereas in the same fibroblasts with impaired cell-substratum adhesion, IGF-I induced tyrosine phosphorylation of FAK (Baron et al., 1998). The effect of IGF-I on the phosphorylation status of Cas, cortactin, tensin has not been reported. It remains unknown whether the phosphorylation of the focal adhesion proteins is affected during IGF-I-stimulated motility in breast epithelial cells.

The aim of the present work was to study how IGF-IR-mediated motility is accomplished and regulated in MCF-7 breast cancer epithelial cells overexpressing the IGF-IR (MCF-7/IGF-IR). We have shown that IGF-IR hyperexpression induced augmented cell migration in vitro, and that the activated IGF-IR promoted development of the membrane protrusion characteristic for motile cells. Initiation of cell motility was associated with coordinated reorganization of the actin cytoskeleton and focal adhesion contacts. Our work provides evidence that IGF-IR signaling triggers a phosphotyrosine phosphatase activity that modulates phosphorylation of focal adhesion proteins FAK, Cas, paxillin, and, thereby, regulates the locomotive functions in MCF-7/IGF-IR cells.

MATERIALS AND METHODS

Cell Culture and Chemicals

Cells were grown in DMEM:F12 (1:1) containing 5% calf serum. To starve of serum, the cells were incubated in phenol red-free serum-free DMEM containing 0.5 mg/ml bovine serum albumin (BSA), 1 μM $FeSO_4$ and 2 mM L-glutamine (SFM) for 24 hours. MCF-7 human breast epithelial cells overexpressing the IGF-IR were derived by stable transfection with pcDNA3/IGF-IR plasmid (Guvakova and Surmacz, 1997a).

BSA, Cytochalasin D (CD), Phenylarsine oxide (PAO), Tetramethylrhodamine B Isothiocyanate (TRITC)-conjugated phalloidin were purchased from Sigma. Human recombinant IGF-I was from BACHEM.

Migration

The migration of cells was assessed in modified Boyden chambers. Briefly, cells were suspended in regular culture medium, and $2x10^4$ of cells were plated into each upper chamber onto the Transwell polycorbanate membrane filter (8 μm pores, Corning Costar). The lower chamber contained either the regular culture medium or the same culture medium supplemented with 50 ng/ml IGF-I used as a chemoattractant. After 3 or 6 hours of incubation at 37°C, cells were wiped out of the upper chamber, and those cells that traversed the membrane were stained with Coomassie Blue and counted. Each experiment was performed at least 3 times with duplicates of each variant. The statistically significant differences between the values were estimated by ANOVA single-factor analysis of variance.

Immunoprecipitation and Immunoblotting.

Cells were lysed in protein lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1mM EGTA, 10% glycerol, 1% Triton X-100, 20 μg/ml aprotinin, 2mM Na orthovanadate, 1 mM phenylmethylsulfonyl fluoride). FAK, Cas and paxillin were precipitated from the cell lysates (500 μg of total protein) with the specific antibodies: an anti-FAK (A-17) polyclonal antibody (pAb) (Santa Cruz Biotechnology), an anti-Cas monoclonal antibody mAb (Transduction Laboratories) and an anti-paxillin mAb (Transduction Laboratories), respectively. Protein-antibody complexes were collected with either protein A- or anti-mouse IgG-agarose beads overnight. Next, the precipitates were washed with HNTG buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), resolved by SDS-polyacrylamide gel electrophoresis, and transferred onto the nitrocellulose filter. Tyrosine phosphorylation and protein levels were assessed by immunoblotting with an anti-phosphotyrosine mAb (PY-20) (Santa Cruz Biotechnology) and the specific antibodies (the same as used for precipitations), respectively. The proteins were visualized by enhanced chemiluminescent detection (Amersham).

Immunofluorescence

Subconfluent cells grown onto a glass coverslip were fixed in 3.7% formaldehyde in PBS for 15 minutes and permeabilized with PBS containing 0.2% Triton X-100 for 5 minutes. To visualize actin filaments (F-actin), cells were stained with TRITC-conjugated phalloidin (1 μg/ml) for 30 minutes, and examined under a Zeiss Axiophot microscope. Changes in the intracellular distribution of FAK, Cas, paxillin were assessed by labeling

with the primary antibodies: an anti-FAK (A-17) pAb (Santa Cruz Biotechnology), a mix of anti-Cas B and F pAbs (gift of Dr. A. H. Bouton, University of Virginia), an anti-paxillin mAb (Transduction Laboratories), correspondingly, for 60 minutes. Primary antibody detection was performed with Lissamine Rhodamine (LRSC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) or Fluorescein Isothiocyanate (FITC)-conjugated goat anti-mouse IgG (CALBIOCHEM). In control, primary antibody was omitted. Some samples were examined with Zeiss Axiovert 100 MRC 600 confocal laser-scanning (Bio-Rad) immunofluorescence microscope. The images of the horizontal middle sections were obtained.

RESULTS

IGF-IR overexpression in MCF-7 cells is associated with enhanced cell migration

To begin our investigation on the role of the IGF-IR in breast epithelial cell motility, we compared migration of MCF-7 cells, expressing moderate level of the endogenous IGF-IR (6×10^4 receptors/cell), versus MCF-7/IGF-IR cells, with an 18-fold overexpression of the wild type IGF-IR. The migration was elevated in the cells with high expression of the IGF-IR (Fig. 1). After 3 hours of incubation in control medium, the number of migrated MCF-7/IGF-IR cells significantly ($p=0.04$) exceeded the correspondent number of the parental cells (3.9-fold increase in average). The difference was even more striking in 6-hours experiments. The average yield of migrated MCF-7/IGF-IR cells exceeded the correspondent number of MCF-7 cells by 6.6-fold ($p=0.01$). The addition of 50 ng/ml IGF-I in a lower chamber led to stimulation of MCF-7 cell migration towards the chemoattractant (2.1 and 2.9-fold increase after 3 and 6 hours, respectively). In contrast, a

positive IGF-I gradient had no stimulatory effect on migration of MCF-7/IGF-IR cells (Fig. 1). To rule out the possibility that the observed enhanced migration of MCF-7/IGF-IR cells was a result of clonal variation, we examined migration of two additional clones of MCF-7cells expressing 0.5×10^6 and 3×10^6 IGF-IR per cell. After 3 hours, the basal migration in these clones was a 4.6 and 2.9-fold increase over that in MCF-7 cells, and by 6 hour it exceeded 6.7 and 3.8-times the basal migration in MCF-7 cells. The excess of IGF-I in the medium either did not improve migration, or slightly inhibited that property (data not shown). In some experiments, in which the regular culture medium was substituted with SFM, the results obtained were similar to that seen in growth medium (data not shown).

IGF-IR activation promotes morphological transition towards a mesenchymal phenotype in MCF-7 cells

To test whether IGF-IR activation induces motility characteristics in attached cells, we examined the morphology of IGF-I-stimulated cells. Subconfluent, serum-starved MCF-7 and MCF-7/IGF-IR cells displayed a characteristic epithelial morphology with apico-basal polarity where the ventral plasma membranes were tightly adherent to the substratum, whereas basal surfaces of adjacent cells were firmly attached to each other (Fig. 2A,B). A dose response assay established that cell monolayers underwent the morphological reorganization in response to 5-50 ng/ml IGF-I (data not shown). The most drastic changes, characterized by disruption of the epithelial sheet, loss of cell polarity, and development of mesenchymal phenotype by the majority of treated cells, occurred in the presence of 50 ng/ml IGF-I (Fig. 2C,D). In a series of experiments, the following

dynamics of the morphological changes was observed. Within the first 15 minutes of IGF-I stimulation, cell-cell contacts became loose and by 60 minutes, the cells partly detached from the plastic surface and slightly rounded up. Between 1 and 4 hours of continuous IGF-I stimulation, numerous membrane ruffles transformed into dynamic lamellipodial advances, the features of the motile cells. By 2-3 hours most of the cells adopted the dendritic morphology and regrouped, forming irregular multicellular clumps. Although IGF-I stimulated the disruption of monolayer architecture in both cell cultures, the extent of the modifications was more pronounced in MCF-7/IGF-IR cells compared to MCF-7 cells (Fig. 2C and D). Therefore, cells overexpressing the IGF-IR were chosen for analysis in all subsequent experiments.

IGF-IR activation modulates the actin cytoskeletal structure in MCF-7/IGF-IR cells

To examine whether IGF-IR activation induces reorganization of the actin cytoskeleton, the F-actin was visualized in serum-starved cells treated with 50 ng/ml IGF-I for various times (Fig. 3A-E). The earliest detectable effect of IGF-I on the actin cytoskeleton was an acute loss of the long actin filaments. Indeed, within 5 minutes of stimulation disappearance of circumferential actin bundles, disassembly of stress fibre filaments, and development of the widespread actin meshwork marked reorganization of the actin cytoskeleton (Fig. 3B). After approximately 15 minutes, the accumulation of F-actin was observed at the cell margins within the structures resembling small membrane ruffles and microspikes (Fig. 3C,D). Between 1 and 4 hours, when the cells developed multiple extended protrusions, the fine long actin filaments reappeared, traversing cytoplasm and ending up the in veil-like lamellipodia.

To address the question whether the actin polymerization and/or re-assembly might be involved in the development of IGF-I-induced cell protrusions, the following experiments were performed. In the subconfluent cells, the disassembly of F-actin was artificially induced by pretreatment with 1 μM CD for 5 minutes (compare Fig. 3A and F). Next, the cells were washed and incubated with 50 ng/ml IGF-I alone or IGF-I plus 1 μM CD for an extra hour before recording of the results. In control experiments, the cells were incubated in either SFM or SFM containing 1 μM CD for 1 hour. Recovery in SFM resulted in re-assembly of the fine actin stress fibres (Fig. 3G). The continuous presence of IGF-I in SFM during the recovery caused considerably more prominent F-actin staining than that observed in SFM alone. The long actin filaments spanned the cytoplasm, whereas the short bundles of F-actin were concentrated in membrane protrusions (Fig. 3H). Interestingly, under condition excluding actin polymerization, IGF-I recovery in the presence of CD, the cells did not develop lamellipodial extensions, however, submembraneous short actin bundles were associated into fine "star"-shaped structures that filled the cytoplasm (Fig. 3I). In control, the increased length of exposure to CD alone caused severe disruption of F-actin structures and retraction of plasma membrane at the cell edges (Fig. 3J).

IGF-IR stimulation changes the localization of paxillin-containing focal contacts.

To determine the relationship between dynamic changes in the actin filament network and the focal adhesion contact organization, the conventional immunofluorescence microscopy was performed in adherent MCF-7/IGF-IR cells. Co-staining for paxillin and actin revealed that paxillin clustering, typically seen in serum-starved cells, disappeared

when stress fibres disassembled within 5-15 minutes of IGF-I stimulation (compare Fig. 4A,B and C,D). Approximately 1 hour after IGF-I addition, when the fine actin filaments re-appeared, the elongated streaks of stained paxillin were observed in numerous membrane protrusions (compare Fig. 4A,B and E,F).

To investigate whether changes in paxillin localization paralleled to the intracellular distribution of the other focal adhesion-associated proteins such as FAK and Cas, confocal immunofluorescence microscopic analysis was performed in unstimulated and IGF-I-stimulated MCF-7/IGF-IR cells. Samples of subconfluent cells were doubly stained with an anti-paxillin mAb (Fig. 5A(a)-F(a)) and one of the following pAb either an anti-FAK (Fig. 5A(b),C(b),E(b)) or an anti-Cas (Fig. 5B(b),D(b),F(b)). Serum-starved cells were fully spread and adherent to substratum. In these cells, paxillin staining was detectable as punctuated arrays of dots along the edges of cells and fine clusters distributed over the ventral surface of the cells, indicating the positions of numerous focal adhesion contacts. FAK and Cas were partially co-localized with paxillin (Fig. 5A,B). Within 15 minutes of IGF-I stimulation, paxillin as well as FAK and Cas staining diffused into the cytoplasm. Typically, at this time point, cells were slightly rounded-up and had only a few paxillin-positive focal contacts (Fig. 5C,D). These results agreed with the overall loss of the paxillin plaque staining obtained with conventional fluorescence microscopy. In a period of 1-4 hours cells formed lamellipodial extensions, where numerous streaks of stained paxillin were localized. FAK and Cas staining could not be clearly detected in the cell membrane protrusions (Fig. 5E(b),F(b)). The intracellular redistribution of FAK was additionally visualized with the 2A7 mAb raised against C-

terminus of pp125 FAK (gift from Dr. J. T. Parsons, University of Virginia). FAK staining was found to be similar to that observed with an anti-FAK(A-17) pAb.

Activation of the IGF-IR induces rapid tyrosine dephosporylation of focal adhesion-associated proteins: FAK, Cas and paxillin

Actin fibres dissassembly was shown to correlate with the reduction of FAK tyrosine phosphorylation in CHO cells (Knight et al., 1995). We examined FAK tyrosine phosporylation status in unstimulated and IGF-I-stimulated MCF-7/IGF-IR cells. In serum-starved cells, FAK was prominently tyrosine phosphorylated (Fig. 6A'). Within 5 minutes of IGF-I treatment, FAK became dephosphoryated by 50% at average, (as judged by scanning densitometry in three independent experiments) (Fig. 6A). As shown on the graph and the representative blot, the level of FAK phosphorylation remained low for at least 15 minutes, then elevated to almost basal level by 1 hour (Fig. 6A and A', upper panel). We further considered the possibility that tyrosine dephosphorylation might inactivate FAK catalytic activity, which in turn could alter the content of phosphorylated tyrosines in FAK target(s). Consequently, Cas and paxillin were immunoprecipitated from the same lysates as FAK. In control serum-starved cells, tyrosine phosphorylation of both proteins was high (Fig. 6B',C', upper panels). After 5 minutes of IGF-I stimulation, the Cas tyrosine phosphorylation was reduced by more than 70 % of control level. Tyrosine dephosphorylation of Cas was transient, as within 15 minutes of IGF-I addition its phosphorylation was noticeably elevated, and by 1 hour it almost reverted back to the basal level (Fig. 6B,B'). Within 5 minutes of IGF-I stimulation, paxillin became dephosphorylated on tyrosine residues by 50%. Over the next 10 minutes, the paxillin

tyrosine phosphorylation declined further and sustained at the low level (30-40% of basal phosphorylation) for at least 4 hours (Fig. 6,C). The protein levels among the samples of each of the precipitated protein were comparable at the studied time points, as shown when the anti-phosphotyrosine blots were stripped and re-probed with the appropriate antibodies (Fig. 6A',B',C,' lower panels).

In control experiment, to investigate the pattern of activation of the IGF-IR signaling molecules, we analyzed the kinetics of protein tyrosine phosphorylation in the whole cell lysates (Fig. 7A). Within 5 minutes IGF-I stimulated prominent phosphorylation of two bands of 175 kDa and 95 kDa, corresponding to IRS-1 and the IGF-IRβ subunit, respectively. In parallel, IGF-I reduced tyrosine phosphorylation of the band of 120-130 kDa, which contained FAK and Cas, and was heavily phosphorylated at time point zero. The kinetics of MAPK activity perfectly correlated with the phosphotyrosine profile of the IGF-IR/IRS-1 complex (Fig. 7B).

PAO prevents IGF-I-induced tyrosine dephosphorylation of FAK, Cas and paxillin, blocks development of lamellipodia and inhibits migration of MCF-7/IGF-IR cells in vitro

To investigate whether tyrosine dephosphorylation of FAK, Cas and paxillin is required for IGF-I-induced motility, we attempted to block a putative IGF-I-induced PTPase. For this purpose a trivalent arsenical compound PAO was selected. PAO has been shown to inhibit tyrosine phosphatase activity in insulin signaling and up-regulate tyrosine phosphorylation of FAK and paxillin in fibroblasts (Noguchi et al., 1994; Retta et al., 1996).

First, to examine whether PAO affects the phosphorylation status of the IGF-IR signaling molecules, we analyzed the pattern of phosphotyrosine proteins as well as MAPK activity in PAO-treated MCF-7/IGF-IR cells. Serum-starved cells were incubated with either 5 μM PAO or SFM for 10 minutes and than stimulated with 50 ng/ml IGF-I for 5 minutes. Blots of the whole lysates were hybridized with either anti-phosphotyrosine or anti-active MAPK antibodies. As seen in Fig. 8A, PAO did not affect IGF-I-induced phosphorylation of IGF-IR and IRS-1, as well as activation of MAPK, whereas it effectively prevented dephosphorylation of 120-130 kDa band. We next examined the effect of PAO on the IGF-I-induced dephosphorylation of focal adhesion proteins. Pretreatment of MCF-7/IGF-IR cells for 10 minutes with 5 μM PAO before stimulation with 50 ng/ml IGF-I abolished dephosphorylation of Cas, essentially blocked dephosphorylation of FAK, however, did not prevent dephosphorylation of paxillin (Fig. 8B). The time course analysis revealed that PAO-mediated rescue of focal adhesion proteins from dephosphorylation was dependent on the duration of PAO pretreatment (data not shown). Pre-incubation with PAO for 60 minutes resulted in notably increased basal tyrosine phosphorylation of FAK and Cas (Fig. 8C. lane 3). After the stimulation of these cells with IGF-I for 5 minutes (Fig. 8C, lane 4) or longer (Fig. 8C, lane 5 and 6), tyrosine phosphorylarion of FAK, Cas and paxillin was sustained.

We further tested whether PAO affects IGF-I-induced changes in cell morphology and motility. The subconfluent MCF-7/IGF-IR cells were incubated with 5 μM PAO for 60 minutes to reassure the block of tyrosine phosphorylation of FAK, Cas and paxillin, while control cells were kept in SFM for 60 minutes (Fig. 9A and D, respectively). Next, both cell monolayers were simultaneously stimulated with 50 ng/ml IGF-I, and the

morphology of the cells was recorded after 15 and 60 minutes (Fig. 9B,E and C,F). After 60 minutes, PAO-treated cells formed lobopodia-like membrane advances resembling those displayed by serum-starved cells in response to IGF-I after 15 minutes of treatment (compare Fig. 9C and E). Interestingly, PAO did not inhibit the development of the actin-rich membrane ruffles, as confirmed by staining with rhodamine-phalloidin (data not shown). However, the outward extensions of lamellipodia, typically seen in control cells after 60 minutes of IGF-I addition, appeared to be blocked, thereby suggesting at least a partial inhibition of morphological transition towards the mesenchymal phenotype. Notably, the shorter (10 minutes) pretreatment with 5 μM PAO, when paxillin dephosphorylation still occurred did not prevent the phenotypic transformation (data not shown).

As we demonstrated earlier in this study, MCF-7/IGF-IR cells exhibited increased migration in vitro. To assay the effect of PAO on migration, the cells in suspension were treated with various concentrations of PAO for 5 minutes prior to plating onto the filter membrane. The scoring of the cells that traversed a porous filter after 6 hours revealed a dose-dependent inhibition of migration in PAO-treated cells. The maximal reduction by 80%, at average, was determined in cells pretreated with 5 μM PAO (Fig. 10). Prolonged pre-incubation for 15 and 30 minutes with 1 μM PAO revealed a time dependent inhibition of cell migration (data not shown).

DISCUSSION

In this study, we investigated how motility is accomplished and regulated in MCF-7 human breast cancer epithelial cells generated to express high level of the IGF-IR. For the

first time we demonstrated that, in breast cancer epithelial cells, increased expression of the IGF-IR correlated with augmented cell migration in vitro. The IGF-IR overexpression per se was sufficient to promote the advanced locomotive abilities of the cells, thereby allowing them to bypass IGF-I requirements. Activation of the IGF-IR correlated with the initiation of cell motility in polarized cells that in response to IGF-I acquired the phenotypic characteristics of the motile cells, by developing the multiple membrane protrusions and assuming the fibroblast-like shape. The extent of phenotypic transformation was more pronounced in MCF-7/IGF-IR than in the parental MCF-7 cells. Therefore, we concluded that amplified IGF-IR signaling was necessary to convert epithelial cells from polarized morphology to a motile phenotype, and that lammelipodial formation correlated with increased cell motility.

In the current paper, we closely analyzed actin cytoskeletal reorganizations in MCF-7/IGF-IR cells following 4 hours of IGF-I stimulation, and identified that activation of the IGF-IR caused dynamic multiphasic restructuring of the actin filamentous network, which normally supported polarization of mammary epithelial cells. The first rapid phase of this process was characterized by cytoplasmic accumulation of the short actin bundles, presumably due to acute dissasembly of the long actin filaments, which, in serum-starved cells, were arranged in circumferential actin bundles and stress fibres. One possibility is that the rapid breakdown of the actin filaments implicates the activation of the actin-severing proteins, like gelsolin, whose stimulation, in turn, requires the transient increase of intracellular Ca^{2+} (Burtnick et al., 1997). Whether a rise in Ca^{2+} and activation of the actin-controlling proteins does occur following IGF-IR activation, remains to be determined. Most of the activated receptor tyrosine kinases, including the IR, bind

phospholipase C γ (PLC γ) (Kayali et al., 1998), utilizing the ubiquitous pathway through hydrolysis of phosphoinositides and release of intracellular Ca^{2+} from endoplasmic reticulum (Clapham, 1995). Despite the large homology between the IR and the IGF-IR, direct coupling of PLC γ and the IGF-IR has not been confirmed (Seely et al., 1995). Alternatively, it is possible that the IGF-IR may utilize another, as yet unidentified mechanisms controlling the level of the intracellular Ca^{2+}. In this respect, it is interesting that recent experiments revealed the ability of the activated IGF-IR to increase Ca^{2+} entry into the cell via up-regulation of calcium-permeable cation channels on the cell surface (Kanzaki et al., 1997).

In IGF-I-stimulated MCF-7/IGF-IR cells, the short initial phase of F-actin disruption was changed to accumulation of condensed F-actin along the cell periphery in about 15 minutes. The latter process was followed by re-appearance of the fine long actin filaments traversing the cytoplasm and ending in the newly formed membrane advances. Extension of the membrane protrusions apparently required actin polymerization, since an addition of CD, the drug that caps the plus-end of actin filament and blocks actin polymerization, prevented lamellipodial extension. In many cell types, actin polymerization is closely linked with activation of the PI3-kinase signaling pathway (Wennström et al., 1994; Lange et al., 1998). We have previously shown that IGF-I-induced tyrosine phosphorylation of the IGF-IR and IRS-1 as well as IGF-I-stimulated PI3-kinase activity were higher in MCF-7/IGF-IR than in the parental cells (Guvakova and Surmacz, 1997a,b). Hence, PI3-kinase activity triggered by amplified IGF-IR signaling is likely to be involved in actin polymerization, development of membrane protrusions, and subsequently initiation of MCF-7/IGF-IR cell motility. It is interesting in

this context, that direct PI3-kinase activation was shown to be sufficient to disrupt epithelial polarization and induce motility in mammary epithelial cells (Keely et al., 1997).

Our results also suggest that apart from regulating actin polymerization, activation of IGF-IR signaling may lead to specific re-assembly of the short actin bundles. Indeed, in cells treated with IGF-I and CD simultaneously, the short actin filaments re-associated into the fine "star"-shaped structures. We speculate that the IGF-IR activates the actin filament cross-linking proteins, possibly those that have been localized to membrane protrusions (Ridley, 1994). This hypothesis is currently being tested.

In the present work, we have demonstrated that in parallel to reorganization of the actin cytoskeleton, activation of the IGF-IR promoted the intracellular redistribution of the focal adhesion proteins such as FAK, Cas and paxillin. In spread static cells, FAK and Cas were partially co-localized with paxillin to focal adhesion plaques of typical for the epithelial cell arrangement (DePasquale et al., 1994). In partially rounded cells, appearing shortly after IGF-I stimulation, actin stress fibres disassembled, and the characteristic "arrowhead"–shaped paxillin clusters were virtually absent. In these cells, paxillin as well as FAK and Cas were stained in tiny dots diffused in cytoplasm that might reflect a loss of stable cell-substratum adhesion due to disruption of the actin-focal adhesion links in cells preparing to move. In the migrating cells, the numerous elongated streaks of paxillin were distributed along the fine actin filaments in newly forming membrane protrusions. However, we observed no clearly detectable co-staining of FAK and Cas with paxillin at this time point. Therefore, the activation of IGF-IR signaling resulted in structural remodeling of the paxillin-rich focal contacts and re-localization of paxillin into the

developing membrane protrusions. Since paxillin possesses multiple protein-protein interaction motifs (Turner and Miller, 1994), it is tempting to speculate that paxillin plays a key role in directing structural and regulatory molecules into the dynamic membrane advances promoting thereby motility of breast epithelial cells.

In the present report, we have begun elucidation of the molecular mechanisms underlying IGF-IR-mediated motility, and have provided evidence for the regulatory role of the IGF-IR on phosphotyrosine content of the focal adhesion proteins. Firstly, we found that activation of the IGF-IR triggers an acute and transient tyrosine dephosphorylation of FAK that might temporarily switch off this major catalytic element of the focal adhesion complex. Additionally, our results provide the first evidence that Cas, a component of focal adhesions, undergoes IGF-I-dependent tyrosine dephosphorylation. This protein contains one SH3 domain and multiple putative SH2- and SH3-domain binding sites, and is known to be stably associated with FAK and Src family kinases (Polte and Hanks, 1997). The kinetic profile of the Cas dephosphorylation is almost identical to that of FAK, consistent with the data that FAK plays a role, either directly or indirectly via the activation of Src-family kinases, in the tyrosine phosphorylation of Cas (Tachibana et al., 1997). Alternatively, the IGF-R could activate a protein tyrosine phosphatase (PTPase) controlling the function of either FAK, or Cas, or both focal adhesion proteins. In this respect it is interesting that SH3 domain of Cas directly binds to proline-rich region of PTP1B, the cytoplasmic PTPase, which is also known to interact with the IR (Liu et al., 1996; Seely et al., 1996). However, at present the regulatory role of PTP1B in either IR or IGF-IR signaling remains obscure. In accordance with the idea that dephosphorylation and thereby enzymatic inactivation of

FAK down regulates tyrosine phosphorylation of its targets, we have found that another FAK-associated phosphoprotein paxillin, a central structural element of the focal adhesions, underwent remarkable tyrosine dephosphorylation in response to IGF-I. The delayed time course of the paxillin dephosphorylation, relatively to FAK, and sustained low level of the paxillin tyrosine phosphorylation for at least 4 hours pointed to indirect effect of FAK on paxillin, presumably through a focal adhesion-associated PTPase. The recent discovery of the physical association between a nonreceptor tyrosine phosphatase PTP-PEST and paxillin suggests that paxillin itself or other paxillin-binding partners, including FAK, might serve as PTP-PEST substrates. (Shen et al., 1998). Although there is no evidence for the link between IGF-IR signaling and PTP-PEST function, these peculiar findings provide the intriguing opportunities for the future investigations. Another, and probably the more enticing candidate on a role of IGF-IR-regulated PTPase; which controls the function of the focal adhesion proteins particularly on FAK, is PTP1D. Firstly, this cytolpasmic PTPase has been shown to mediate insulin-induced dephosphorylation of FAK and paxillin in CHO cells overexpressing the IR (Ouwens et al., 1996). Secondly, PTP1D via its SH2 domains can directly bind to the activated IR and IGF-IR, or to their major substrate IRS-1 and that, in turn, could result in stimulation of its PTPase activity (Rocchi et al., 1996). Furthermore, its association, at least, with IRS-1 is transient: it reaches maximum after 1 minute of insulin stimulation, and then subsides by 5 minute (Noguchi et al., 1994). Additionally, this PTPase plays a positive role in mediating mitogenic MAPK pathway (Milarski and Satiel, 1994). Moreover, the recent studies identified an important role of PTP1D (also known as Shp2/SH-PTP2/Syp) in regulating cell spreading, migration, and cytoskeletal architecture in fibroblasts,

presumably via control of FAK (Yu et al., 1998). All these evidences are in agreement with our observations on the function of a putative IGF-IR-activated PTPase. Nevertheless, in our preliminary experiments, we were unable to detect IGF-I-induced activation of PTP1D in MCF-7/IGF-IR cells, possibly because of the low expression of the PTPase in these cells.

Over the last decade a list of newly discovered PTPases has been substantially expanded. Recently, the expression of 31 different PTPases was reported in human breast cancer cell line ZR75-1 (Keane et al, 1996). The certain PTPases including LAR (Leukocyte common antigen-related) and FAP-1 (Fas-associated PTP-1) are involved in down regulation of the IGF-IR signaling (Zhang et al, 1996; Freiss et al., 1998). Although both PTPases are expressed in MCF-7 cells, they are unlikely candidates on a role of a PTPase regulating focal adhesion proteins in IGF-I-stimulated cells. Likewise, MKP-1, a dual-specificity phosphatase implicated in dephosphorylation of MAPK in vivo (Neel and Tonks, 1997), can not be rapidly activated in response to IGF-I.

Despite unknown identity of IGF-IR-induced PTPase in MCF-7/IGF-IR cells, the critical role of a putative PTPase in initiation of breast epithelial cell motility is strongly supported by our experiments with PAO, a phosphotyrosine phosphatase inhibitor. PAO-pretreatment prevented FAK, Cas and more importantly paxillin from IGF-I-induced tyrosine dephosphorylation, and that was sufficient to block development of the motile membrane protrusions in polarized cells, and to inhibit cell migration in vitro. These findings propose the IGF-IR as the upstream regulator of a PAO-sensitive PTPase, which participates, through yet unknown mechanism, in controlling FAK, Cas and paxillin tyrosine phosphorylation.

It has been previously suggested that tyrosine phosphorylation of FAK, Cas and paxillin is critical for formation of different signaling complexes with the adaptor molecules such as Grb2, Crk, Nck that may direct signal progression through Ras/Raf/MAPK pathway (Schlaepfer et al., 1997; Ishiki et al., 1997). Here we provide evidence that the remarkable tyrosine dephosphorylation of FAK, Cas and paxillin in response to IGF-I did not affect IGF-I-induced MAPK activation.

In summary, we have established that increased IGF-IR expression promoted locomotive abilities in MCF-7 breast cancer epithelial cells in vitro. Activation of IGF-IR signaling resulted in initiation of motility in polarized cell through the series of structural changes in the actin filamentous network and paxillin-rich focal contacts. The IGF-IR-mediated motile response required a putative PAO-sensitive PTPase activity, acting upstream of focal adhesion proteins, particularly FAK, Cas and paxillin.

We surmise that amplified IGF-IR signaling in breast cancer epithelial cells expressing high level of the IGF-IR allows bypassing of the normal ligand-dependent control of locomotive functions, thereby providing the cells with an advantage in spreading and migration during breast cancer development. This hypothesis is an agreement with the striking quantitative prevalence and enhanced autophosphorylation of the IGF-IR observed in malignant versus normal human breast tissues (Resnik et al., 1998). Further elucidation of the mechanisms of IGF-IR-mediated motility in vitro and in vivo will be a key to understanding of IGF-IR involvement in breast cancer.

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FIGURE LEGENDS

Fig. 1. Overexpressed IGF-IR promotes increased migration of MCF-7 cells. Cell migration was assessed as the described in Materials and Methods. The cell suspension in regular culture medium was plated into the upper chamber. The lower chamber contained either the regular culture medium (control) or the same culture medium supplemented with 50 ng/ml IGF-I, used as chemoattractant (+IGF-I). Presented data are the means of several independent experiments. Error bars show s.e.m., n=3. Asterisks indicate the statistically significant differences between the MCF-7 and MCF-7/IGF-IR cell values estimated by ANOVA ($P<0.05$).

Fig. 2. IGF-I stimulates formation of lamellipodial extentions in MCF-7/IGF-IR cells. The representative phase-contrast micrographs show morphology of the subconfluent serum-starved MCF-7 (A) and MCF-7/IGF-IR (B) cells. In C, MCF-7 and in D, MCF-7/IGF-IR serum-starved cells were stimulated with 50 ng/ml IGF-I for 4 hours. Scale bar, 100 μm.

Fig. 3. IGF-I stimulation rapidly changes F-actin pattern in MCF-7/IGF-IR cells. The representative images show MCF-7/IGF-IR cells, in which F-actin was visualized with TRITC-labeled phalloidin. On the left panel: serum-starved cells (A); cells stimulated with 50 ng/ml IGF-I for 5 minutes (B), for 15 minutes (C), for 60 minutes (D) and for 4 hours (E). On the right panel: serum-starved cells exposed to 1 μM CD for 5 minutes (F), CD-pretreated cells recovered in SFM for 1 hour (G), CD-pretreated cells recovered in 50 ng/ml IGF-I in SFM for 1 hour (H), CD-pretreated cells recovered in 50 ng/ml IGF-I plus 1 μM CD in SFM for 1 hour (I), serum-starved cells exposed to 1 μM

CD in SFM for 1 hour (J). In I, arrows show the position of "star"-shaped F-actin accumulations. Scale bar, 20 μm.

Fig. 4. Actin cytoskeleton reorganization is accompanied by redistribution of paxillin following IGF-I stimulation of MCF-7/IGF-IR cells. The representative images show the cells co-stained with an anti-paxillin mAb (A,C,E) and TRITC-phalloidin (B,D,F). Serum-starved cells (A,B). Cells treated with 50 ng/ml IGF-I for 5 minutes (C,D) and for 60 minutes (E,F). Arrows in A,C,E point to the position of the paxillin accumulations in cell protrusions. Scale bar, 20 μm.

Fig. 5. IGF-I stimulation changes intracellular localization of paxillin, FAK and Cas in MCF-7/IGF-IR cells. The representative confocal microscopy images demonstrate cells co-labeled with an anti-paxillin mAb (A(a),C(a),E(a)) and an anti-FAK (N-17) pAb (A(b),C(b),E(b)); cells co-labeled with an anti-paxillin mAb (B(a),D(a),F(a)) and a mix of anti Cas (B+F) pAb (B(b),D(b),F(b)). In A and B, serum-starved cells; in C and D, cells treated with 50 ng/ml IGF-I for 15 minutes and in E and F, for 60 minutes are shown. The representative areas of coincident staining of paxillin with either FAK or Cas are marked by arrowheads in A and B, respectively. In E and F, arrowheads indicate the accumulation of paxillin in prolonged streaks localized to membrane protrusions. Scale bar, 20 μm.

Fig. 6. IGF-I induces rapid tyrosine dephosphorylation of FAK, Cas and paxillin in MCF-7/IGF-IR cells. The graphs in A,B,C represent the time courses of the relative tyrosine phosphorylation of FAK, Cas and paxillin in response to 50 ng/ml IGF-I, correspondingly. The intensity of the bands of the phosphorylated proteins was measured by laser scanning densitometry reading. The value of tyrosine phosphorylation for each protein in serum-starved cells (time point 0 min) was taken as 100%. Error bars show

s.e.m., n=4. In A',B',C' the representative blots demonstrate the levels of the tyrosine phosphorylated FAK, Cas and paxillin (upper panels) and the amount of these proteins in the correspondent samples (lower panels) before and after IGF-I stimulation at the indicated time points.

Fig. 7. IGF-I differentially modulates protein tyrosine phosphorylation in MCF-7/IGF-IR cells. In A, the representative anti-phosphotyrosine blot of the whole lysates (20 μg of total protein per lane) of MCF-7/IGF-IR cells stimulated with 50 ng/ml IGF-I for indicated times is shown. On the left: protein Mr marker; on the right: arrows indicate the positions of IRS-1 and IGF-IRβ subunit, confirmed by stripping of this blot and re-probing firstly with an anti-IRS-1 pAb (UBI), and then with an anti-IGF-IRβ pAb (Santa Cruz Biotechnology). An open arrowhead indicates the position of the tyrosine-dephosphorylated band in IGF-I-treated cells. In B, the representative anti-active MAPK blot illustrates the typical kinetics of MAPK activation in MCF-7/IGF-IR cells stimulated with 50 ng/ml IGF-I. The level of the activated MAPK (Erk1 and Erk2) was measured in the whole cell lysates (20 μg of protein per sample) by immunoblotting with an anti-active MAPK pAb (Promega).

Fig. 8. PAO prevents tyrosine dephosphorylation of focal adhesion proteins in cells stimulated with IGF-I. (A) Upper blot is the representative anti-phosphotyrosine blot of the whole lysates of MCF-7/IGF-IR cells pretreated with either SFM alone or SFM containing 5 μM PAO for 10 minutes, and then stimulated with 50 ng/ml IGF-I for 5 minutes. On the left: protein Mr marker; on the right: arrows indicate the positions of IRS-1 and IGF-IRβ subunit, confirmed as described under Fig. 7. An open arrowhead indicates the position of the dephosphorylated band in IGF-I-treated cells. In a lower

panel, positions of Erk1 and Erk2 are indicated in anti-active MAPK blot. (B) Blots show tyrosine phosphorylarion and protein levels in the FAK, Cas and paxillin immunoprecipitates from serum-starved cells pre-treated with either SFM or 5 μM PAO for 10 minutes, and then stimulated with 50 ng/ml IGF-I for 5 minutes. (C) Tyrosine phosphorylarion and protein levels of the immunoprecipitated FAK, Cas and paxillin in the samples of serum-starved cells pre-treated with either SFM or 5 μM PAO for 60 minutes, and then stimulated with 50 ng/ml IGF-I for indicated times.

Fig. 9. PAO blocks morphological transitions in MCF-7/IGF-IR cells stimulated with IGF-I. The representative phase-contrast micrographs show morphology of MCF-7/IGF-IR cells under different conditions. Serum-starved cells were pretreated with 5 μM PAO for 60 minutes (A), and then stimulated with 50 ng/ml IGF-I for either 15 minutes (B) or 60 minutes (C). In parallel, serum-starved cells (D) were stimulated with 50 ng/ml IGF-I for either 15 minutes (E) or 60 minutes (F). Scale bar, 100 μm.

Fig. 10. PAO inhibits migration of MCF-7/IGF-IR cells in a dose-dependent manner. Cell migration was assessed as described in Materials and Methods. Cells were incubated in culture medium in the absence (control) or presence of the indicated concentrations of PAO for 5 minutes. Next, cells were washed and re-suspended in regular medium, plated on the filter, and incubated for 6 hours. The lower chamber contained regular culture medium. The number of the control migrated cells was taken as 100%. The percentage of the migrated PAO-pretreated cells was estimated relatively to control. Abscissa: Concentration of PAO in μM. Ordinate: Relative migration. The result is an average of two experiments with duplicates.

Figure 1

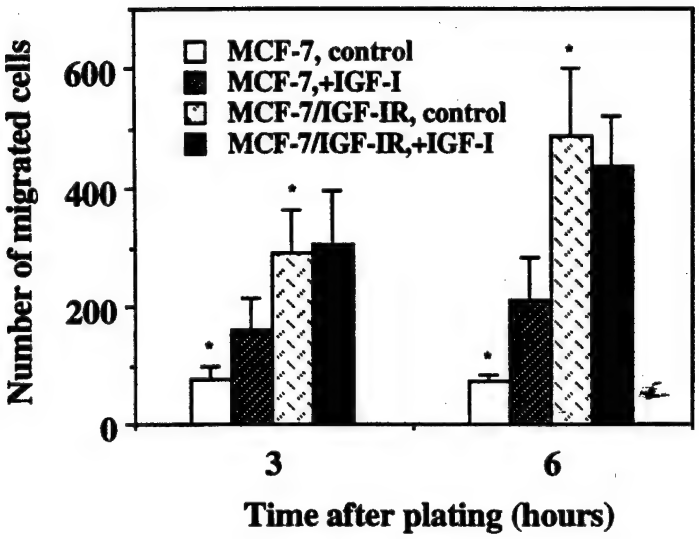

MCF-7, control
MCF-7,+IGF-I
MCF-7/IGF-IR, control
MCF-7/IGF-IR,+IGF-I
Number of migrated cells
600
400
200
0
3
6
Time after plating (hours)

Figure 2

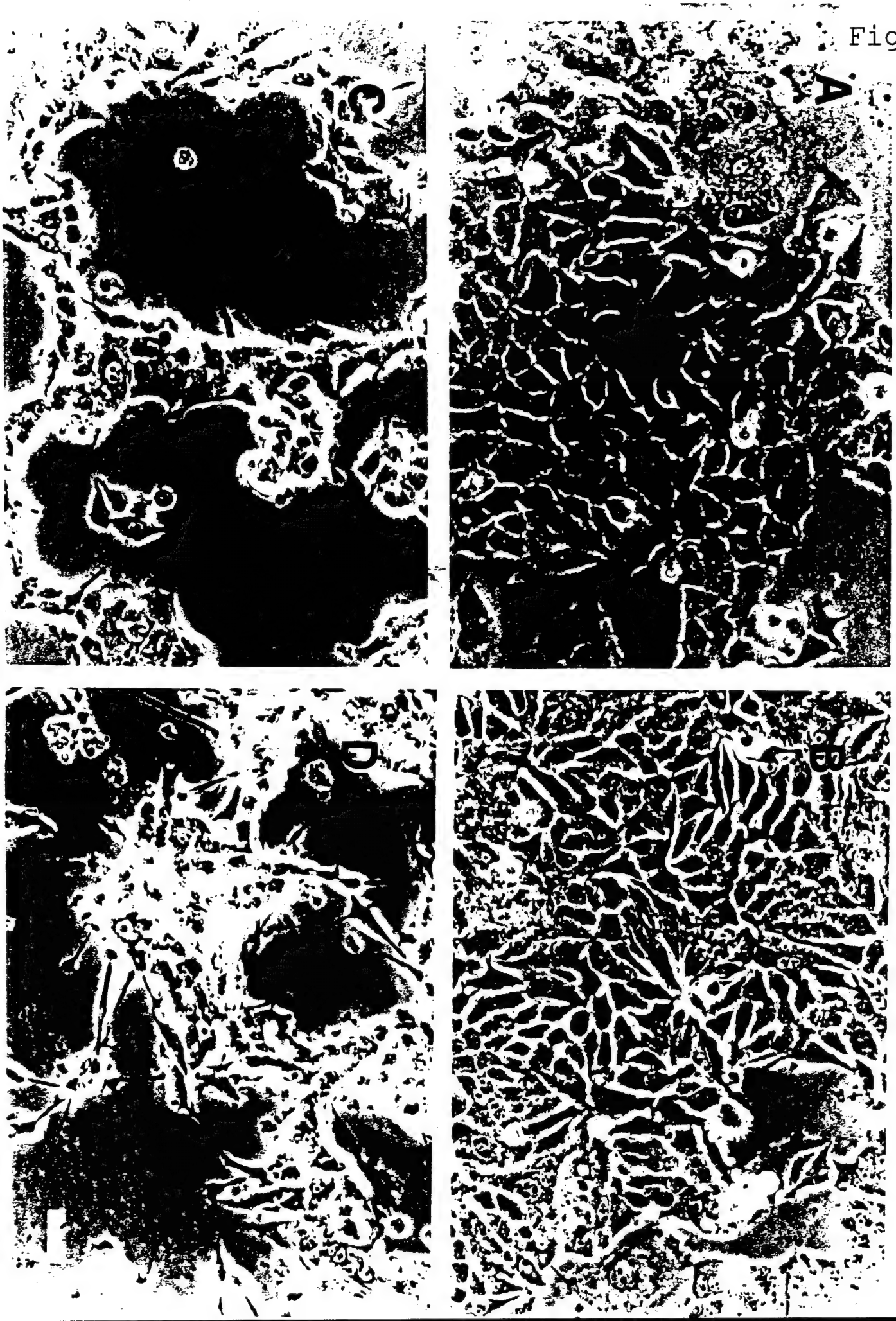


Figure 3

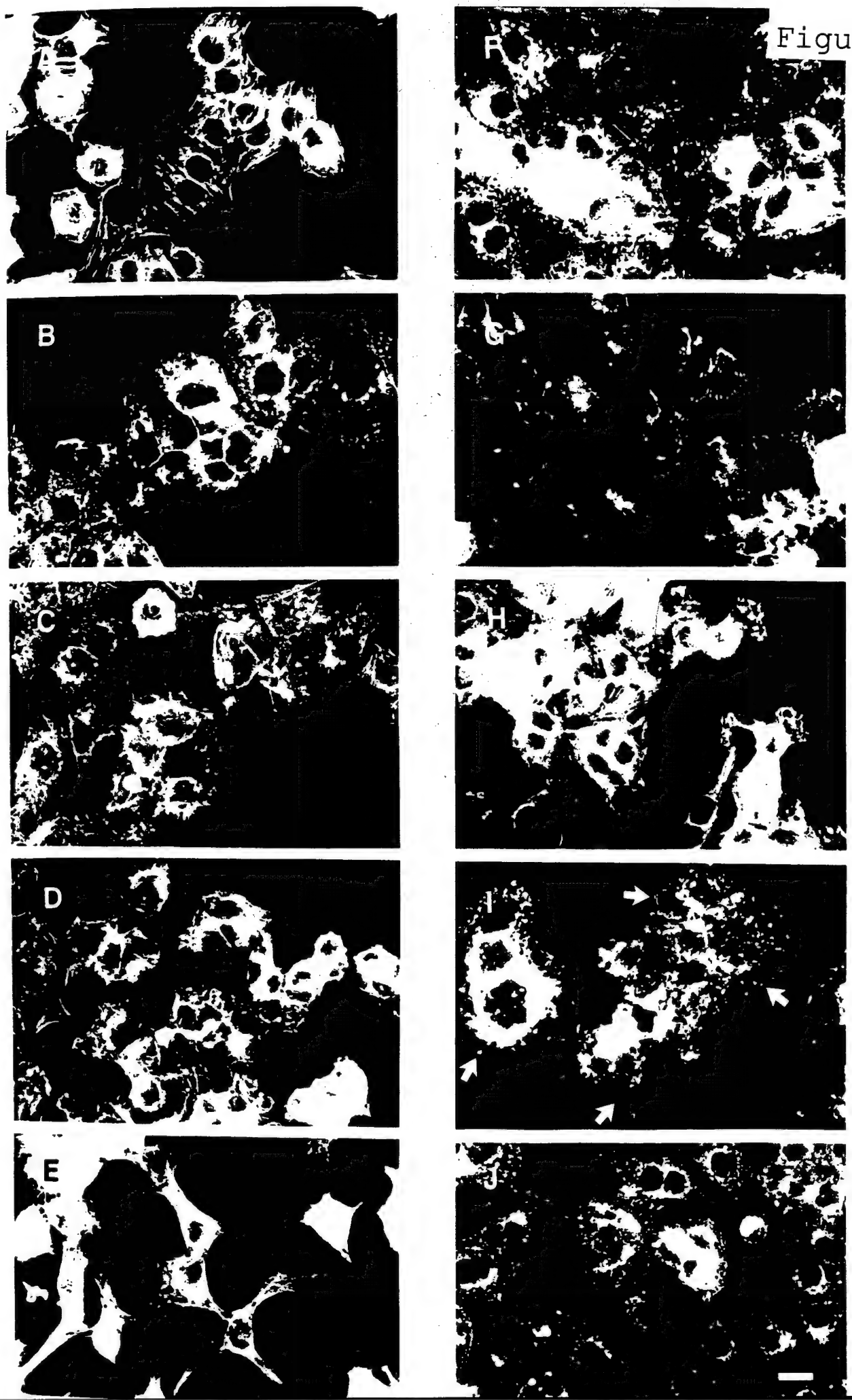


Figure 4

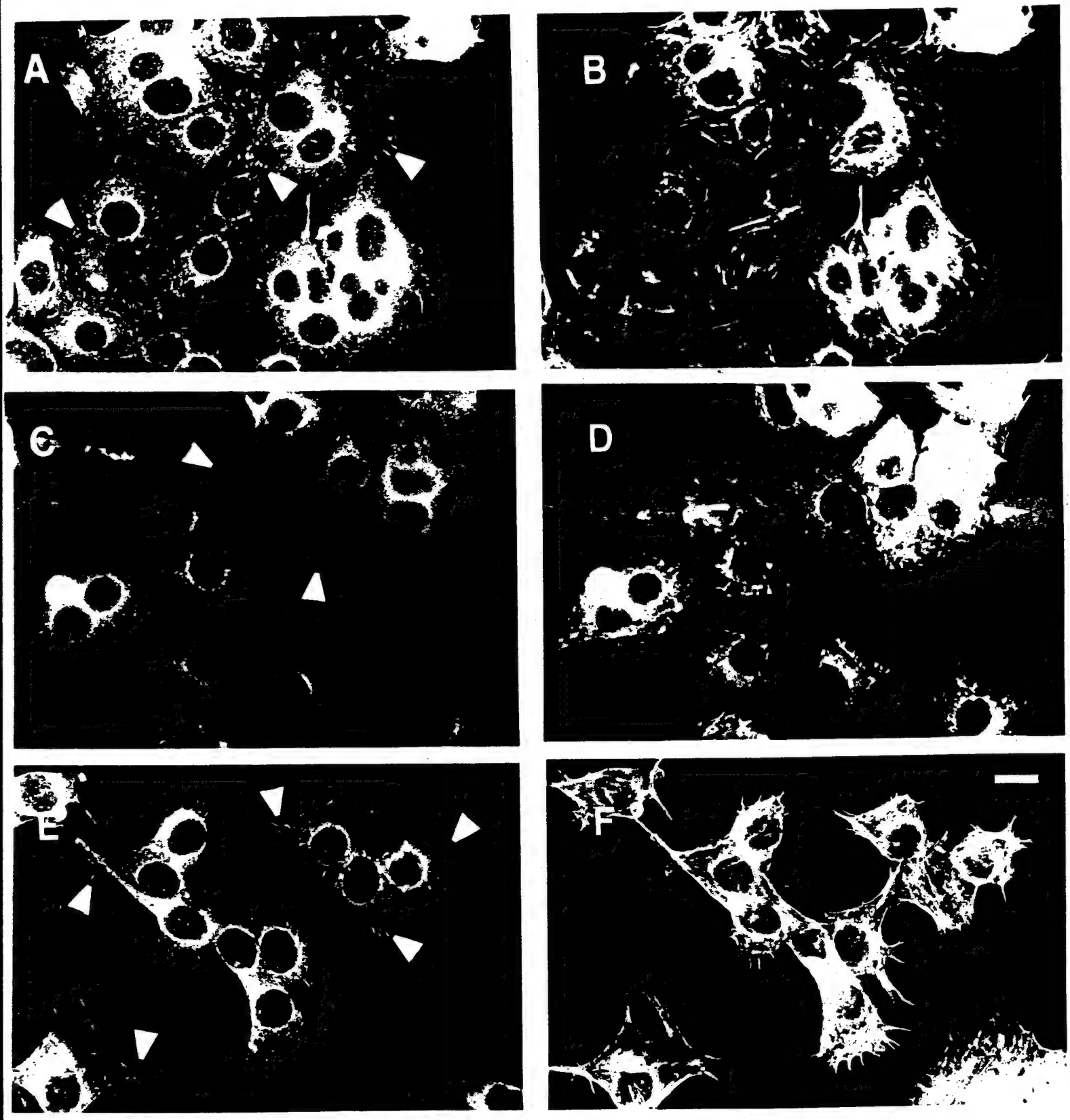


Figure 5

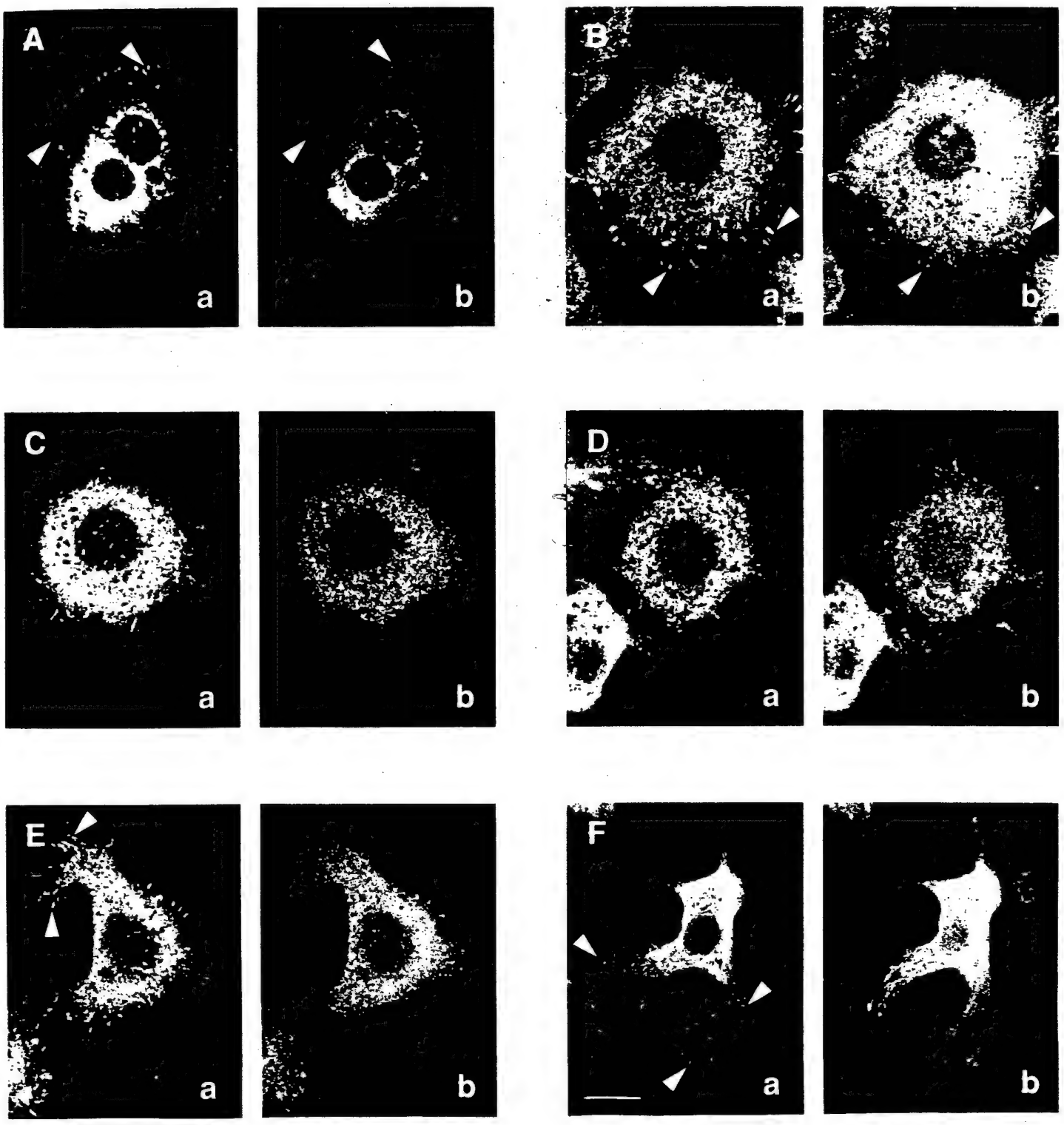


Figure 6

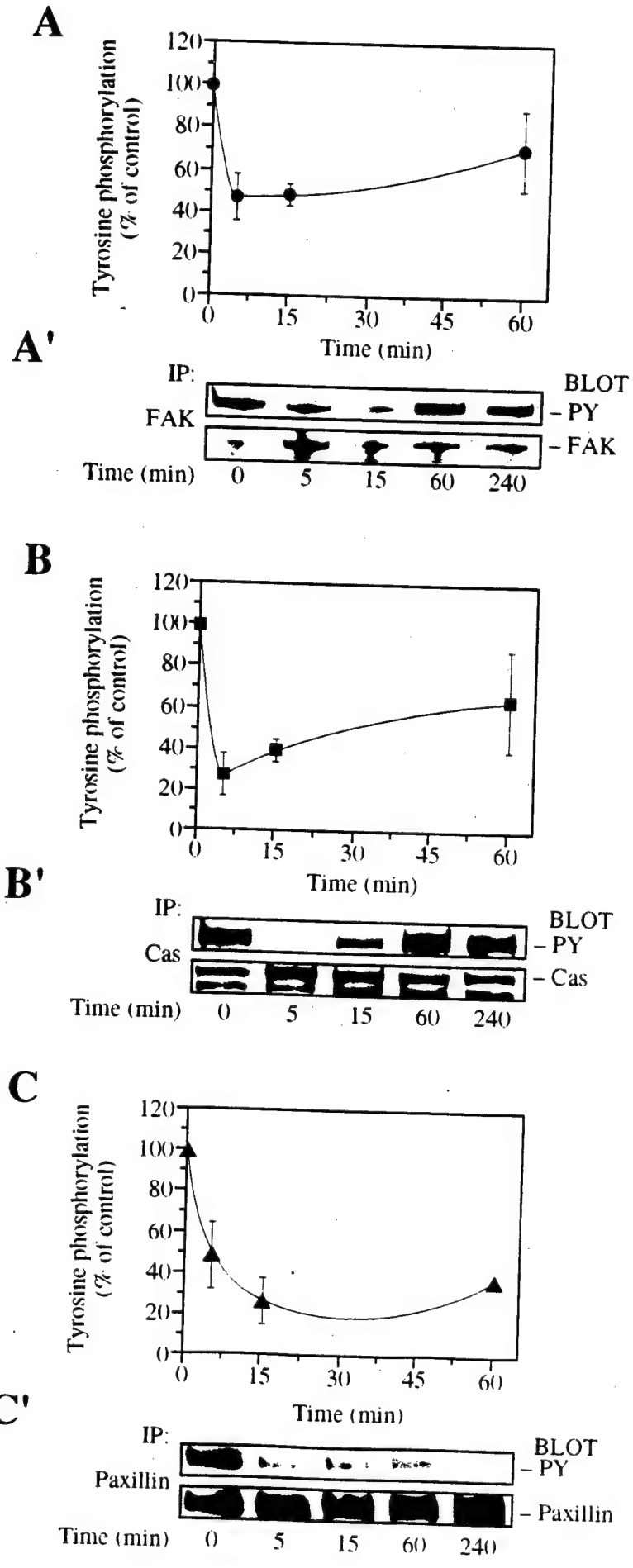

A
Tyrosine phosphorylation (% of control)
120
100
80
60
40
20
0
0
15
30
45
60
Time (min)
A'
IP:
FAK
BLOT
– PY
– FAK
Time (min)
0
5
15
60
240
B
Tyrosine phosphorylation (% of control)
Time (min)
B'
IP:
Cas
BLOT
– PY
– Cas
Time (min)
C
Tyrosine phosphorylation (% of control)
Time (min)
C'
IP:
Paxillin
BLOT
– PY
– Paxillin
Time (min)

Figure 7

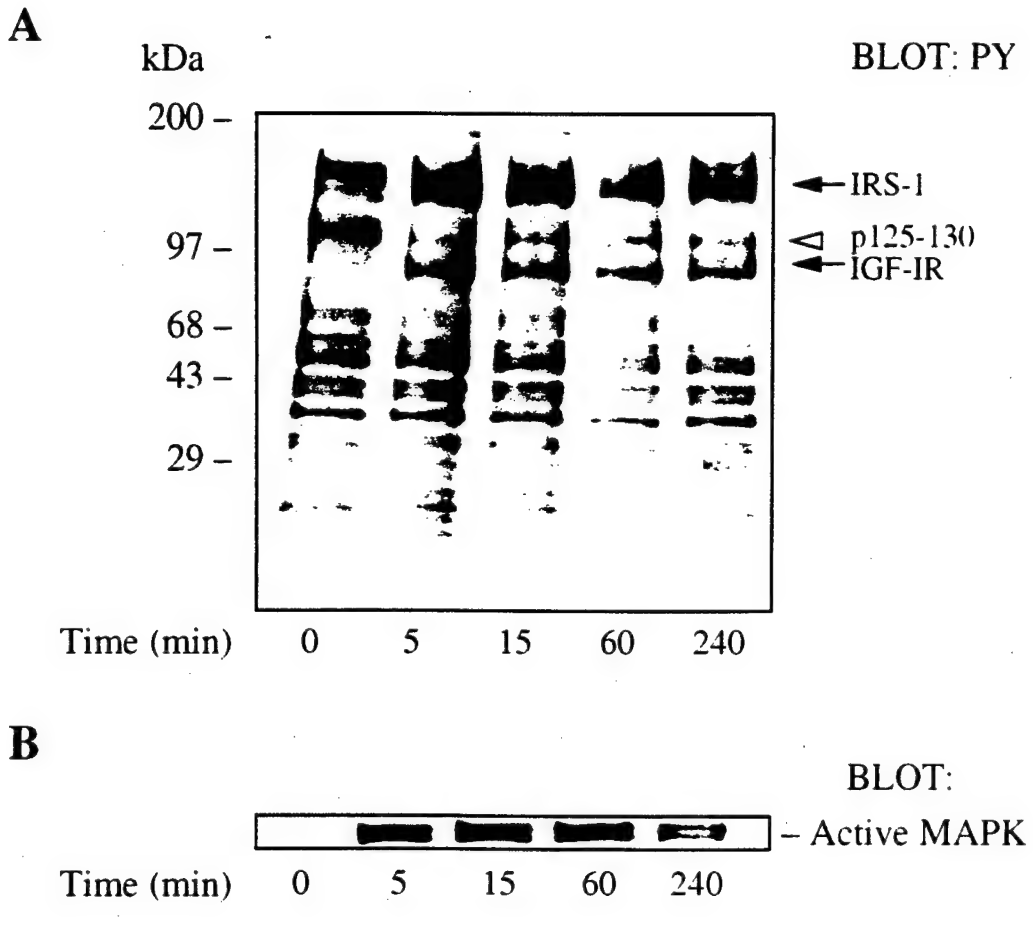

A
kDa
BLOT: PY
200 –
97 –
68 –
43 –
29 –
IRS-1
p125-130
IGF-IR
Time (min) 0 5 15 60 240
B
BLOT:
– Active MAPK
Time (min) 0 5 15 60 240

Figure 8

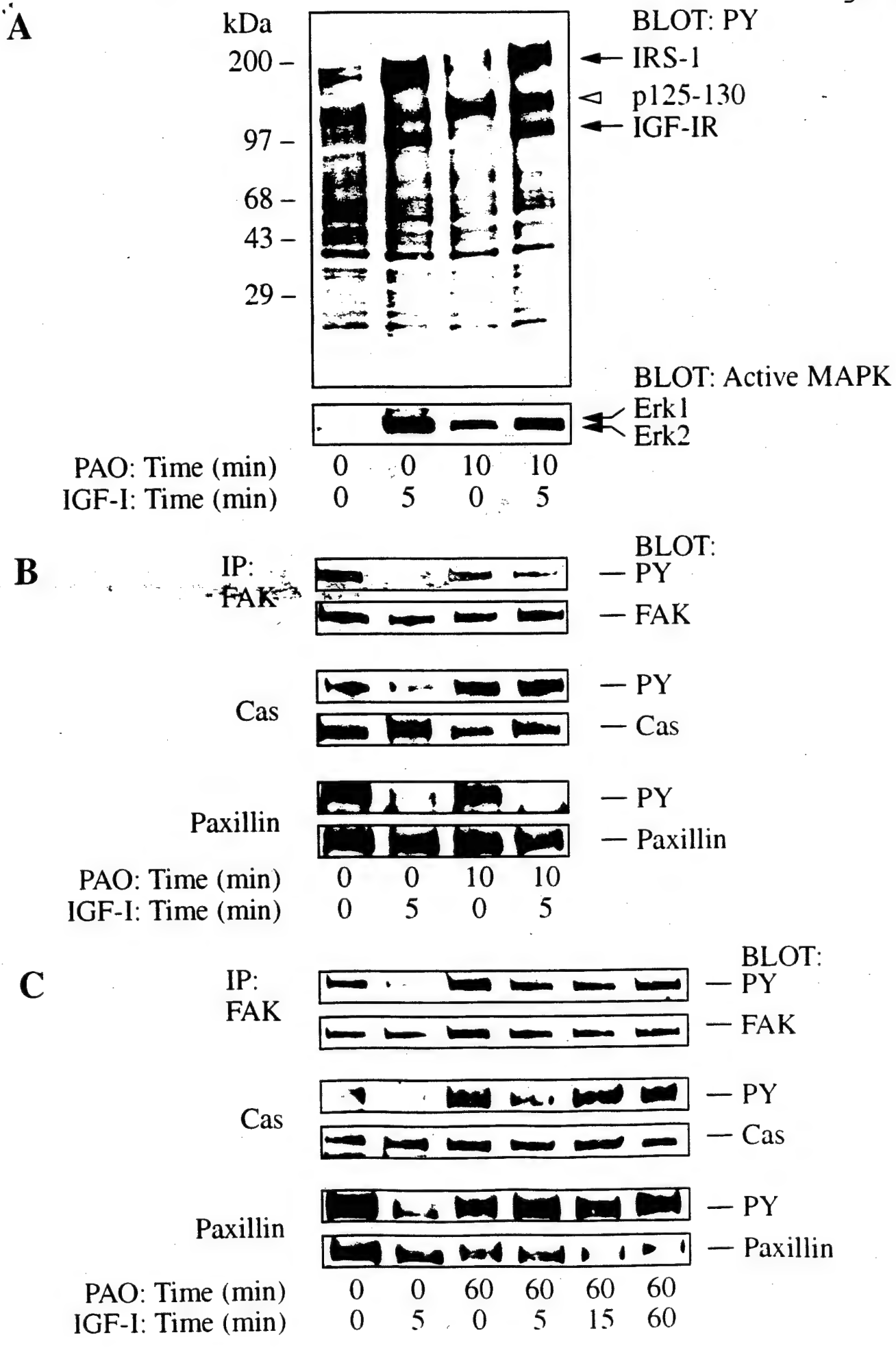

A
kDa
200 –
97 –
68 –
43 –
29 –
BLOT: PY
IRS-1
p125-130
IGF-IR
BLOT: Active MAPK
Erk1
Erk2
PAO: Time (min) 0 0 10 10
IGF-I: Time (min) 0 5 0 5
B
IP: FAK
BLOT:
— PY
— FAK
Cas
— PY
— Cas
Paxillin
— PY
— Paxillin
PAO: Time (min) 0 0 10 10
IGF-I: Time (min) 0 5 0 5
C
IP: FAK
BLOT:
— PY
— FAK
Cas
— PY
— Cas
Paxillin
— PY
— Paxillin
PAO: Time (min) 0 0 60 60 60 60
IGF-I: Time (min) 0 5 0 5 15 60

Figure 9

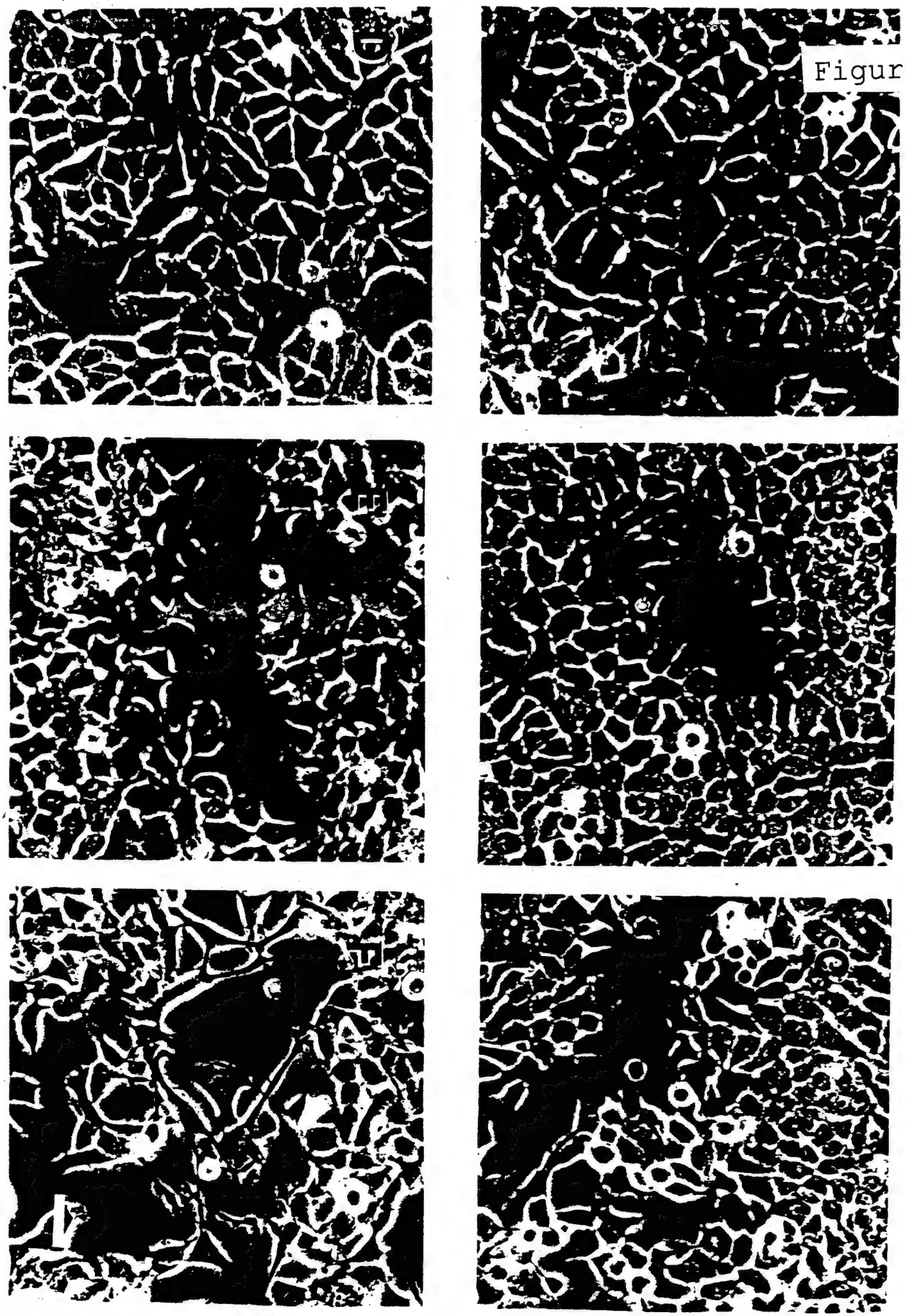
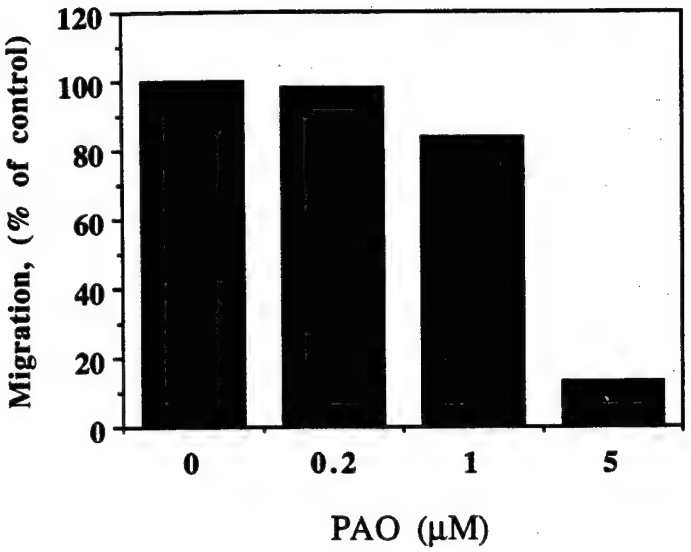


Figure 10



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INSULIN RECEPTOR SUBSTRATE 1 IS A TARGET FOR THE PURE ANTIESTROGEN ICI 182,780 IN BREAST CANCER CELLS

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The pure antiestrogen ICI 182,780 inhibits insulin-like growth factor (IGF)-dependent proliferation in hormone-responsive breast cancer cells. However, the interactions of ICI 182,780 with IGF-I receptor (IGF-IR) intracellular signaling have not been characterized. Here, we studied the effects of ICI 182,780 on IGF-IR signal transduction in MCF-7 breast cancer cells and in MCF-7-derived clones overexpressing either the IGF-IR or its 2 major substrates, insulin receptor substrate 1 (IRS-1) or src/collagen homology proteins (SHC). ICI 182,780 blocked the basal and IGF-I-induced growth in all studied cells in a dose-dependent manner; however, the clones with the greatest IRS-1 overexpression were clearly least sensitive to the drug. Pursuing ICI 182,780 interaction with IRS-1, we found that the antiestrogen reduced IRS-1 expression and tyrosine phosphorylation in several cell lines in the presence or absence of IGF-I. Moreover, in IRS-1-overexpressing cells, ICI 182,780 decreased IRS-1/p85 and IRS-1/GRB2 binding. The effects of ICI 182,780 on IGF-IR protein expression were not significant; however, the drug suppressed IGF-I-induced (but not basal) IGF-IR tyrosine phosphorylation. The expression and tyrosine phosphorylation of SHC as well as SHC/GRB binding were not influenced by ICI 182,780. In summary, downregulation of IRS-1 may represent one of the mechanisms by which ICI 182,780 inhibits the growth of breast cancer cells. Thus, overexpression of IRS-1 in breast tumors could contribute to the development of antiestrogen resistance. *Int. J. Cancer* 81:299–304, 1999.
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ICI 182,780, an alpha-alkylsulfinylamide, is a new-generation pure antiestrogen (Wakeling *et al.,* 1991). The drug has shown great promise as a second-line endocrine therapy agent in patients with advanced breast cancer resistant to the non-steroidal antiestrogen tamoxifen (Tam). Indeed, in several *in vitro* and *in vivo* studies, the antitumor effects of ICI 182,780 were greater than those of Tam (Nicholson *et al.,* 1996; Osborne *et al.,* 1995; de Cupis *et al.,* 1995; Chander *et al.,* 1993; Wakeling *et al.,* 1991). Moreover, unlike Tam, ICI 182,780 lacks agonist (estrogenic) activity and its administration does not appear to be associated with deleterious side effects such as induction of endometrial cancer or retinopathy (Osborne *et al.,* 1995; Chander *et al.,* 1993). ICI 182,780 antagonizes multiple cellular effects of estrogens by impairing the dimerization of the estrogen receptor (ER) and by reducing ER half-life (de Cupis and Favoni, 1997; Chander *et al.,* 1993). ICI 182,780 also interferes with growth factor-induced growth, but it is not clear whether this activity is mediated exclusively through the ER, or if some ER-independent mechanism is implicated (de Cupis *et al.,* 1995). Despite their great antitumor effects, pure antiestrogens do not circumvent the development of antiestrogen resistance, as most breast tumor cells initially sensitive to ICI 182,780 eventually become unresponsive to the drug (de Cupis and Favoni, 1997; Pavlik *et al.,* 1996; Nicholson *et al.,* 1995). The mechanism of this resistance is not clear, but it has been suggested that both mutations of the ER as well as alterations in growth factor-dependent mitogenic pathways may be involved (de Cupis and Favoni, 1997; Larsen *et al.,* 1997; Pavlik *et al.,* 1996; Wiseman *et al.,* 1993).

The insulin-like growth factor (IGF) system [IGFs, IGF-I receptor (IGF-IR) and IGF binding proteins (IGFBP)] plays a critical role in the pathobiology of hormone-responsive breast cancer (reviewed in Surmacz *et al.,* 1998). In the experimental setting, IGF-IR has been shown to stimulate growth and transformation, improve survival, as well as regulate cell-cell and cell-substrate interactions in breast cancer cells (Surmacz *et al.,* 1998). Moreover, overexpression of different elements of the IGF system, such as IGF-II, IGF-IR or insulin receptor substrate 1 (IRS-1), provides breast cancer cells with growth advantage and reduces or abrogates estrogen growth requirements (Surmacz *et al.,* 1998). On the other hand, downregulation of IGF-IR expression, inhibition of IGF-IR signaling and reduced bioavailability of the IGFs have all been demonstrated to block proliferation and survival as well as to interfere with motility or intercellular adhesion in breast cancer cells (Surmacz *et al.,* 1998).

Clinical studies confirm the role of the IGF-I system in breast cancer development. First, IGF-IR has been found to be up to 14-fold overexpressed in breast cancer compared with its levels in normal mammary epithelium (Surmacz *et al.,* 1998; Resnik *et al.,* 1998; Turner *et al.,* 1997). Moreover, cellular levels of IGF-IR or its substrate IRS-1 correlate with cancer recurrence at the primary site (Rocha *et al.,* 1997; Turner *et al.,* 1997). The ligands of IGF-IR, IGF-I and IGF-II, are often present in the epithelial and/or stromal component of breast tumors, indicating that an autocrine or a paracrine IGF-IR loop may be operative and involved in the neoplastic process (Surmacz *et al.,* 1998). In addition, endocrine IGFs probably also contribute to breast tumorigenesis since the levels of circulating IGF-I correlate with breast cancer risk in premenopausal women (Hankinson *et al.,* 1998).

ICI 182,780 interferes with the IGF-I system in breast cancer cells. The antiestrogen has been shown to attenuate IGF-I-stimulated growth, modulate expression of IGFBPs and downregulate IGF binding sites (Surmacz *et al.,* 1998; de Cupis and Favoni, 1997; de Cupis *et al.,* 1995). The interactions of ICI 182,780 with the IGF-IR signaling pathways, however, have not been characterized.

Our previous work demonstrated that in breast cancer cells, Tam interferes with the IGF-IR signaling acting upon IGF-IR substrates IRS-1 and src/collagen homology proteins (SHC) (Guvakova and Surmacz, 1997). Normally, activation of IGF-IR results in the recruitment and tyrosine phosphorylation of IRS-1 and SHC, followed by their association with several downstream effector proteins and induction of various signaling pathways (Surmacz *et al.,* 1998). For instance, association of either IRS-1 or SHC with GRB-2/SOS complexes activates the Ras/MAP pathway, whereas binding of IRS-1 with p85 stimulates PI-3 kinase. Tam treatment blocks IGF-dependent growth, which coincides with decreased tyrosine phosphorylation of IRS-1 and IGF-IR and with hyperphos-

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phorylation of SHC (Guvakova and Surmacz, 1997). Here, we demonstrate the interactions of ICI 182,780 with IGF-IR signaling and discuss the relevant similarities and differences in the modes of action of the 2 antiestrogens.

MATERIAL AND METHODS

Cell lines and cell culture conditions

We used MCF-7 cells and several MCF-7-derived clones overexpressing either IGF-IR (MCF-7/IGF-IR cells), IRS-1 (MCF-7/IRS-1 cells) or SHC (MCF-7/SHC cells). MCF-7/IGF-IR clone 17 and MCF-7/IRS-1 clones 9, 3 and 18 were developed by stable transfection of MCF-7 cells with expression vectors encoding either IGF-IR or IRS-1 and have been characterized previously (Guvakova and Surmacz, 1997; Surmacz and Burgaud, 1995). MCF-7/SHC cells are MCF-7-derived cells transfected with the plasmid pcDNA3/SHC; compared with MCF-7 cells, the level of $p55^{SHC}$ and $p47^{SHC}$ overexpression in MCF-7/SHC cells is approximately 5-fold (data not shown). The above MCF-7-derived clones express ERs and respond to E2, similar to MCF-7 cells (Guvakova and Surmacz, 1997; Surmacz and Burgaud, 1995). The levels of IRS-1 in MCF-7/IGF-IR and MCF-7/SHC cells are similar to those in MCF-7 cells (see Fig. 2*b* and data not shown).

MCF-7 cells were grown in DMEM:F12 (1:1) containing 5% calf serum (CS). MCF-7-derived clones were maintained in DMEM:F12 plus 5% CS plus 200 μg/ml G418. In the experiments requiring E2-free conditions, the cells were cultured in phenol red-free DMEM containing 0.5 mg/ml BSA, 1 μM $FeSO_4$ and 2 mM L-glutamine (PRF-SFM) (Guvakova and Surmacz, 1997; Surmacz and Burgaud, 1995).

Cell growth assay

Cells were plated at a concentration 2×10^5 in 6-well plates in a growth medium; the following day (day 0), the cells were shifted to PRF-SFM containing different doses of ICI 182,780 (1–300 nM) with or without 50 ng/ml IGF-I and incubated for 4 days. The increase in cell number from day 0 to day 4 in PRF-SFM was designated as 100% growth increase.

IP and WB

The expression and tyrosine phosphorylation of IGF-I signaling proteins were measured by IP and WB, as described previously (Guvakova and Surmacz, 1997; Surmacz and Burgaud, 1995). Protein lysates (500 μg) were immunoprecipitated with the following antibodies (Abs): for the IGF-IR: anti-IGF-IR monoclonal Ab (MAb) alpha-IR3 (Oncogene Science, Cambridge, MA); for IRS-1: anti-C-terminal IRS-1 polyclonal Ab (pAb) (UBI, Lake Placid, NY); for SHC: anti-SHC pAb (Transduction Laboratories, Lexington, KY). Tyrosine phosphorylation was probed by WB with an antiphosphotyrosine MAb PY20 (Transduction Laboratories). The levels of IRS-1, IGF-IR and SHC expression were determined by stripping the phosphotyrosine blots and reprobing them with the following Abs: for IRS-1: anti-IRS-1 pAb (UBI); for IGF-IR: anti-IGF-IR mAb (Santa Cruz Biotechnology, Santa Cruz, CA); for SHC: anti-SHC MAb (Transduction Laboratories). The association of GRB2 or p85 with IRS-1 or SHC was visualized in IRS-1 or SHC blots using an anti-GRB2 MAb (Transduction Laboratories) or an anti-p85 MAb (UBI), respectively. The intensity of bands was measured by laser densitometry scanning.

Northern blotting

The levels of IRS-1 mRNA were detected by Northern blotting in 20 μg of total RNA using a 631 bp probe derived from a mouse IRS-1 cDNA (nt 1351–2002). This fragment (99.8% homology with the human IRS-1 sequence) hybridizes with both human and mouse IRS-1 mRNA (Nishiyama and Wands, 1992).

Statistical analysis

The results in cell growth experiments were analyzed by analysis of variance (ANOVA) or Student's *t*-test, where appropriate.

RESULTS

ICI 182,780 inhibits growth of MCF-7 cells with amplified IGF-IR signaling. Sensitivity to ICI 182,780 is determined by the cellular levels of IRS-1

All cell lines used in this study secrete autocrine IGF-I-like mitogens and are able to proliferate in PRF-SFM (Guvakova and Surmacz, 1997; Surmacz and Burgaud, 1995). The basal (autocrine) growth of the cells was enhanced in the presence of IGF-I (Fig. 1*a,b*). Short (1–2 days) treatments with ICI 182,780 were not sufficient to inhibit cell growth (data not shown), but a 4-day culture in the presence of the antiestrogen produced evident cytostatic effects (Fig. 1*a,b*). In general, the response to ICI 182,780 was dose dependent (Fig. 1*a,b*), however, compared with the other cell lines, the cells highly overexpressing IRS-1 (MCF-7/IRS-1 clones 3 and 18) were more resistant to the drug (Fig. 1*b,c*). Specifically, 1 nM ICI 182,780 inhibited the basal growth by 80, 55 and 50% in MCF-7, MCF-7/IGF-IR and MCF-7/SHC cells, respectively, but the same dose produced only a 20–30% growth inhibition in MCF-7/IRS-1 clones 3 and 18 (Fig. 1*a,b*). Higher concentrations of ICI 182,780 (10 and 100 nM) effectively suppressed the autocrine growth, or even induced cell death in all cell lines, except MCF-7/IRS-1 clone 18, where the maximal reduction (32%) of the basal growth occurred with a 100 nM dose (Fig. 1*b*).

In the presence of IGF-I, the effects of ICI 182,780 were attenuated; 1 nM ICI 182,780 was never cytostatic (data not shown), while the 10 and 100 nM doses inhibited (by 30–50% and 47–78%, respectively) IGF-I-dependent proliferation of cells with low IRS-1 levels (Fig. 1*a,b*). The same doses, however, were less efficient in MCF-7/IRS-1 clones 3 and 18, where growth reduction was 20–25% for 10 nM and 41–47% for 100 nM. Similarly, 300 nM ICI 182,780 produced a prominent cytostatic effect in all cell lines with low IRS-1 expression, but was less active in the clones highly overexpressing IRS-1 (70–93% *vs.* 45–60% growth inhibition) (Fig. 1*a–c*).

The above results suggested that IRS-1 may be an important target for ICI 182,780 action. Consequently, in the next set of experiments we studied the effects of ICI 182,780 on the expression and function of IRS-1.

ICI 182,780 reduces IRS-1 levels and impairs IRS-1 signaling in MCF-7/IRS-1, MCF-7 and MCF-7/IGF-IR cells

In MCF-7/IRS-1 cells grown under basal conditions, IRS-1 was tyrosine phosphorylated for up to 4 days (Fig. 2*a*). IGF-I induced a rapid and marked (5-fold) increase of IRS-1 phosphorylation which persisted for up to 1 day and declined thereafter reaching close to the basal phosphorylation status at day 4. A short ($\leq$1 day) treatment with ICI 182,780 had no consequences on IRS-1 expression or tyrosine phosphorylation. (Fig. 2*a,* panels a and b). However, p85/IRS-1 association was approximately 30% reduced under the basal conditions at day 1 of the treatment (Fig. 2*a,* panel c).

The evident effect of ICI 182,780 action on IRS-1 expression and signaling occurred at day 4, and was especially pronounced in the absence of IGF-I. Specifically, without IGF-I, the drug suppressed IRS-1 protein expression by 60%, which was paralleled by a 60% reduction of IRS-1 tyrosine phosphorylation, and coincided with an almost complete (approximately 95%) inhibition of p85/IRS-1 and GRB2/IRS-1 binding. The addition of IGF attenuated ICI 182,780 action, however, the effects of the treatment remained well detectable: IRS-1 levels were downregulated by 30%, IRS-1 tyrosine phosphorylation by 20% and p85/IRS-1 binding by 30%. Under IGF-I conditions, GRB2/IRS-1 binding was not appreciably affected (Fig. 2*a,* panels a–d).

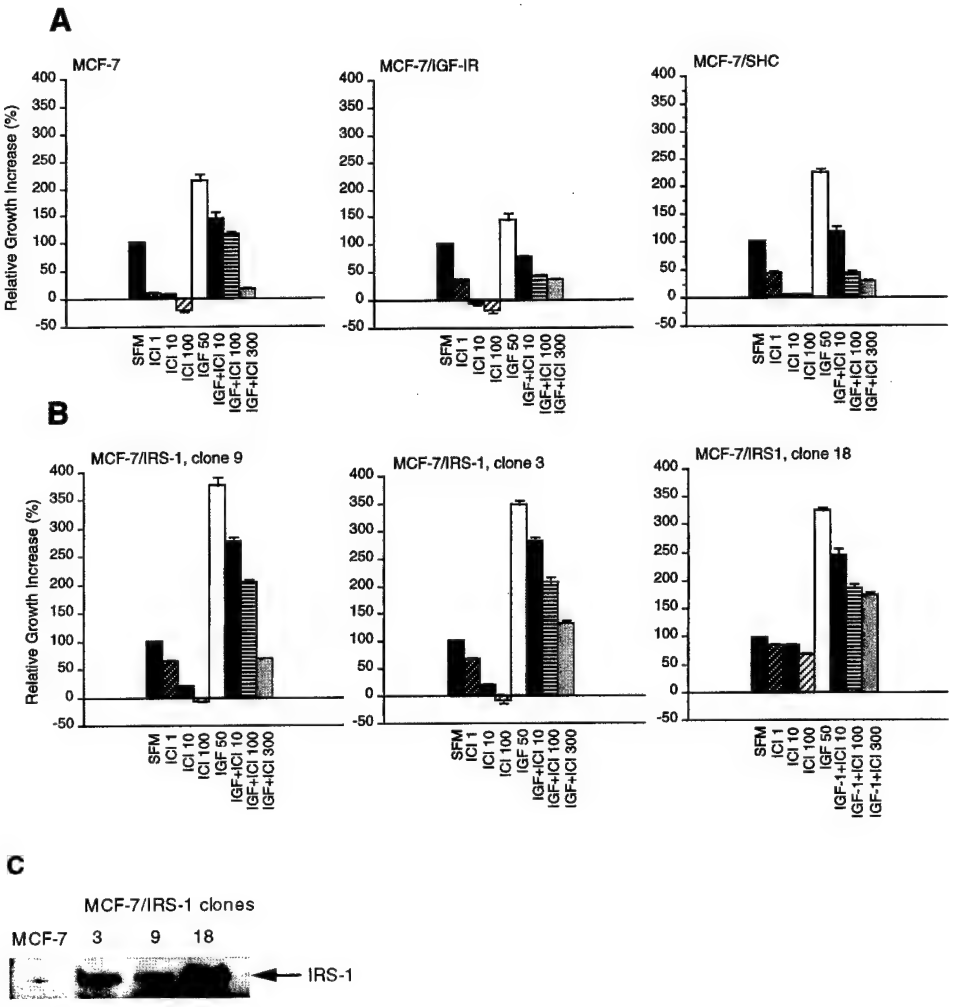

FIGURE 1 – ICI 182,780 inhibits the growth of MCF-7 cells overexpressing different elements of IGF-IR signaling. IRS-1 levels determine ICI 182,780 sensitivity. *(a)* ICI 182,780-induced growth inhibition in the parental MCF-7 cells (8×10^4 IGF-IR/cell), MCF-7/IGF-IR clone 17 (1×10^6 IGF-IR/cell) and MCF-7/SHC (5-fold SHC overexpression over the level in MCF-7 cells). *(b)* Growth reduction in MCF-7/IRS-1 clone 9 (3-fold IRS-1 overexpression over the levels in MCF-7 cells), clone 3 (7-fold overexpression) and clone 18 (9-fold overexpression). The cells were treated with different doses of ICI 182,780 in the presence or absence of 50 ng/ml IGF-I, as described in Material and Methods. The increase in cell number between day 0 and day 4 is taken as 100%. The results are means from at least 4 experiments. Bars indicate standard error. *(c)* Levels of IRS-1 protein in different MCF-7/IRS-1 cell lines. IRS-1 levels were determined by IP and WB as described in Material and Methods. Representative results from 3 experiments are shown.

Importantly, analogous action of ICI 182,780 on IRS-1 expression and tyrosine phosphorylation was seen in other cell lines studied (Fig. 2*b*). In both MCF-7/IGF-IR and MCF-7 cells containing only endogenous IRS-1, ICI 182,780 inhibited the IRS-1 expression under basal conditions by approximately 60%, which was paralleled by the reduced IRS-1 tyrosine phosphorylation (by approximately 90–95%). In the presence of IGF-I, the antiestrogen suppressed the IRS-1 levels by approximately 50% and IRS-1 tyrosine phosphorylation by approximately 40%.

ICI 182,780 attenuates IRS-1 mRNA expression

ICI 182,780 reduced the levels of 5 kb IRS-1 mRNA (Nishiyama and Wands, 1992) in MCF-7 and MCF-7/IGF-IR cells in the absence or presence of IGF-I, by 50 and 70%, respectively (Fig. 3). Moreover, the 5 kb message transcribed from the CMV-IRS-1 plasmid was downregulated (by approximately 70%) in MCF-7/IRS-1 cells treated with both IGF-I and ICI 182,780 (data not shown).

ICI 182,780 inhibits IGF-I-induced but not basal tyrosine phosphorylation of IGF-IR

In MCF-7/IGF-IR cells, IGF-I moderately increased the expression of IGF-IR. This effect was slightly (by 20%) blocked in the presence of ICI 182,780. Under the same conditions, the drug significantly (by 80%) reduced tyrosine phosphorylation of IGF-IR (Fig. 4). ICI 182,780 had no effect on the basal expression of IGF-IR, however, it produced a 30% increase in the basal tyrosine phosphorylation of IGF-IR (Fig. 4). The latter peculiar effect of the antiestrogen occurred in several repeat experiments. Short treatments with ICI 182,780 ($\leq$1 day) were not associated with any significant changes in IGF-IR expression (data not shown).

Long-term ICI 182,780 treatment does not affect SHC signaling

In the presence of IGF-I, SHC tyrosine phosphorylation was moderately induced, with the maximum seen at 1 hr upon stimulation. On the other hand, GRB2/SHC binding peaked at 15 min after IGF-I addition and declined thereafter with the minimal

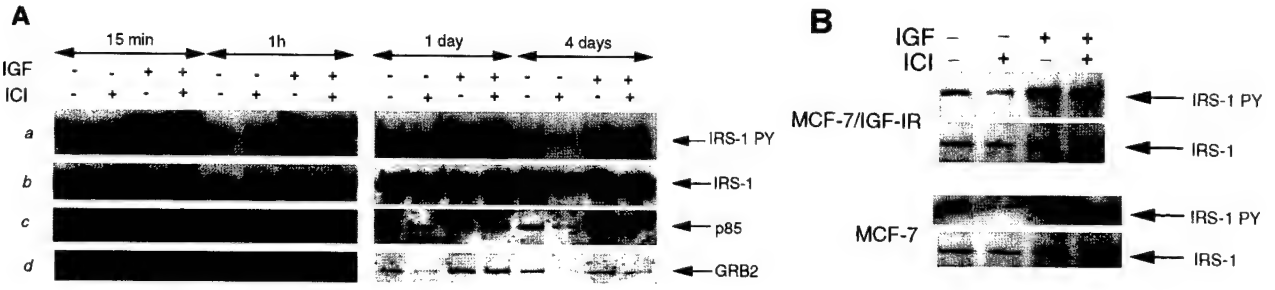

FIGURE 2 – ICI 182,780 inhibits IRS-1-mediated signaling. *(a)* Effects of ICI 182,780 in MCF-7/IRS-1 clone 3. IRS-1 tyrosine phosphorylation (IRS-1 PY) (panel a), protein levels (IRS-1) (panel b), as well as IRS-1-associated p85 of PI-3 kinase (panel c) and GRB2 (panel d) were determined in cells treated for 15 min, 1 hr, 1 day or 4 days with 100 nM ICI 182,780 in the presence or absence of 50 ng/ml IGF-I. In the 1 hr treatment, lane IGF (−) ICI (−) is underloaded. Representative results from 5 experiments are shown. *(b)* Effects of ICI 182,780 on IRS-1 in MCF-7/IGF-IR and MCF-7 cells. IRS-1 tyrosine phosphorylation (IRS-1 PY) and protein levels (IRS-1) were examined in cells treated with 100 nM ICI 182,780 for 4 days. Representative blots of 5 experiments are shown.

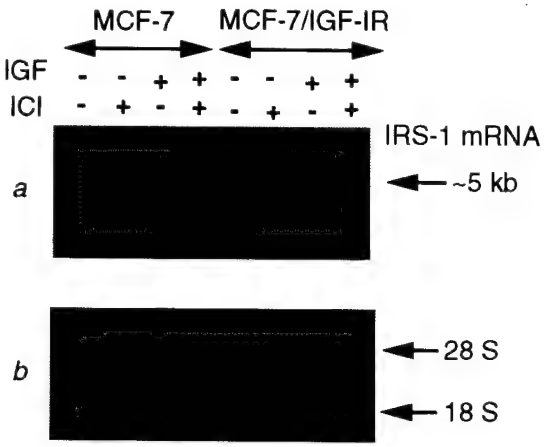

FIGURE 3 – ICI 182,780 attenuates the expression of IRS-1 mRNA levels in MCF-7 and MCF-7/IGF-IR cells. The expression of IRS-1 mRNA was determined in cells treated with 100 nM ICI 182,780 for 4 days in the presence or absence of IGF-I. Panel *a*. IRS-1 mRNA of approximately 5 kb. Panel *b*. Control RNA loading: 28S and 18S RNA in the same blot.

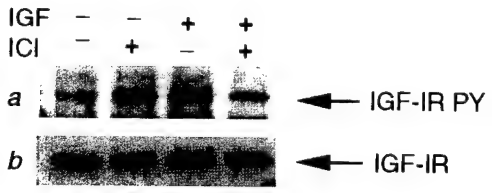

FIGURE 4 – Effects of ICI 182,780 on IGF-IR. IGF-IR tyrosine phosphorylation (IGF-IR PY) (panel *a*) and protein levels (IGF-IR) (panel *b*) in MCF-7/IGF-IR clone 17 treated for 4 days with 100 nM ICI 182,780 in the presence or absence of 50 ng/ml IGF-I. Representative results of 3 different experiments are shown.

binding found at day 4 (Fig. 5). ICI 182,780 treatment, in the presence or absence of IGF-I, failed to induce significant changes in the levels or tyrosine phosphorylation of SHC proteins, except a transient stimulation of the basal SHC tyrosine phosphorylation at 15 min (Fig. 5). Importantly, at all time points, SHC/GRB2 association was not influenced by the drug.

Interestingly, at day 4, SHC tyrosine phosphorylation and SHC/GRB2 binding were suppressed in the presence of IGF-I (Fig. 5). This characteristic regulation of SHC by IGF-I, documented by us previously in MCF-7 cells and MCF-7-derived clones, was not affected by ICI 182,780 (Guvakova and Surmacz, 1997).

Similar lack of ICI 182,780 effects on SHC expression and signaling was noted in MCF-7 and MCF-7/IGF-IR cells (data not shown).

DISCUSSION

Pure antiestrogens have been shown to interfere with one of the most important systems regulating the biology of hormone-dependent breast cancer cells, namely, the IGF-I system (de Cupis and Favoni, 1997; Nicholson *et al.,* 1996). The compounds inhibit IGF-induced proliferation, which is associated with, *i.e.,* downregulation of IGF binding sites and reduction of IGF availability. Similar action has been ascribed to non-steroidal antiestrogens such as Tam or 4-OH-Tam (Chander *et al.,* 1993).

The effects of pure antiestrogens on the IGF signal transduction have been unknown. Here, we studied if and how ICI 182,780 modulates the IGF-IR intracellular pathways in breast cancer cells. We focused on the relationship between drug efficiency and signaling capacities of IGF-IR or IRS-1 since these molecules appear to control proliferation and survival in breast cancer cells (Surmacz *et al.,* 1998; Rocha *et al.,* 1997; Turner *et al.,* 1997).

Previously we found that the cytostatic action of Tam involves its interference with IGF signaling pathways. In particular, Tam suppressed tyrosine phosphorylation of IRS-1 and caused hyperphosphorylation of SHC (Guvakova and Surmacz, 1997). The most important conclusion of the present work is that inhibition of IRS-1 expression is an important element of ICI 182,780 mode of action. The first observation was that amplification of IGF signaling did not abrogate sensitivity to ICI 182,780. Next, ICI 182,780 appeared to affect a specific IGF signaling pathway, as the efficiency of the drug was dictated by the cellular levels of IRS-1, but not that of SHC or IGF-IR. For instance, MCF-7/IGF-IR clone 17 was very sensitive to ICI 182,780 despite a 12-fold IGF-IR overexpression, whereas MCF-7/IRS-1 clones 3 and 18 (7- and 9-fold IRS-1 overexpression, respectively) were quite resistant to the drug (Fig. 1). Moreover, ICI 182,780 reduced IRS-1 levels and tyrosine phosphorylation in several cell lines in the presence or absence of IGF-I, while its action on IGF-IR was limited to the inhibition of IGF-I-induced tyrosine phosphorylation and its effects on SHC were none.

The reduction of IRS-1 expression by ICI 182,780 occurred in all cell lines studied, however, it was clearly more pronounced in the cells expressing lower (endogenous) levels of the substrate (*i.e.,* MCF-7 and MCF-7/IGF-IR cells) (Fig. 2). This suggests that downregulation of IRS-1 by ICI 182,780 is a saturable process, and overexpression of IRS-1 may provide resistance to the drug. Indeed, although we did not notice a strict correlation between

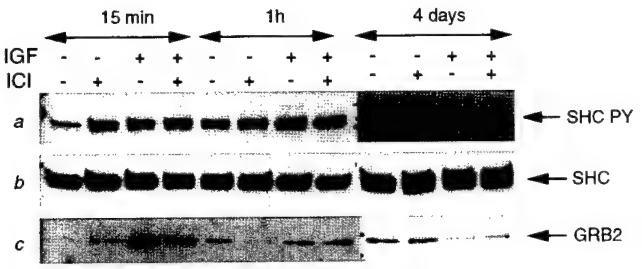

FIGURE 5 – Effects of ICI 182,780 on SHC signaling. SHC tyrosine phosphorylation (SHC PY) (panel *a*), protein levels (SHC) (panel *b*) and SHC-associated GRB2 (panel *c*) were studied in MCF-7/SHC cells treated for 4 days with 100 nM ICI 182,780 in the presence or absence of 50 ng/ml IGF-I. Representative results of 5 experiments are shown.

IRS-1 levels or IRS-1 tyrosine phosphorylation and ICI 182,780-dependent growth inhibition, IRS-1-overexpressing cells tended to be more resistant to the cytostatic action of the antiestrogen (Fig. 1). Interestingly, overexpression of IRS-1 clearly had a greater impact on the response to high doses of ICI 182,780 (≥100 nM) than on the effects of low drug concentrations. This suggests that ICI 182,780 action is multiphased, with the initial inhibition being IRS-1 independent (but perhaps, ER-dependent) and the strong growth reduction associated with the blockade of IRS-1 function (Figs. 1, 2).

ICI 182,780 affected IRS-1 expression not only on the level of protein but also on the level of mRNA. In our experiments, the antiestrogen reduced the expression of IRS-1 mRNA in the presence or absence of IGF-I. However, the mechanism by which ICI 182,780 interferes with IRS-1 mRNA expression was not studied here and it remains speculative. Regarding transcriptional regulation, no estrogen-responsive elements have been mapped in the IRS-1 promoter, but it cannot be ruled out that ICI 182,780 acts indirectly through some other regulatory sequences in the 5′ untranslated region of *IRS-1* gene, such as AP1, AP2, Sp1, C/EBP, E box (Araki *et al.,* 1995; Matsuda *et al.,* 1997). A post-transcriptional component may be argued by the fact that the inhibition of IRS-1 mRNA by ICI 182,780 was evident in IGF-I-treated MCF-7/IRS-1 cells, in which the majority of IRS-1 message originated from the expression plasmid devoid of any IRS-1 promoter sequences (CMV-driven IRS-1 cDNA) (Surmacz and Burgaud, 1995) (data not shown). In addition, the finding that ICI 182,780 similarly inhibited IRS-1 mRNA levels under the basal and IGF-I conditions, but IRS-1 protein was significantly more reduced in the absence of IGF-I (Fig. 3 *vs.* Fig. 2*a*) could suggest that the drug acts upon some IGF-I-dependent mechanism controlling mRNA stability, translation, or post-translational events. In fact, in other experimental systems, IGF-I or insulin regulated various messages, including IRS-1 mRNA, on the post-transcriptional level (Araki *et al.,* 1995).

In its action on IRS-1, ICI 182,780 appeared more potent than Tam, which decreased tyrosine phosphorylation of IRS-1 but did not cause any detectable changes in IRS-1 expression. Our results with Tam suggested that this antiestrogen may influence the activity of tyrosine phosphatases (PTPases) (Guvakova and Surmacz, 1997). Indeed, both Tam and ICI 182,780 interfere with IGF-I-dependent growth by upregulating PTPases LAR and FAP-1 (Freiss *et al.,* 1998). In the present work, ICI 182,780 effects on phosphatases acting on IRS-1 were impossible to assess, since the drug also affected IRS-1 expression (Fig. 3). However, some interference of ICI 182,780 with the phosphorylation/dephosphorylation events could be indicated, for instance, by our experiments with IGF-IR, where, under basal conditions, the compound induced IGF-IR phosphorylation without evident modifications of the receptor expression (Fig. 4).

Other important observations stemming from our results concern similarities and differences between the effects of ICI 182,780 and Tam on the IGF-IR and SHC. While Tam did not modulate the expression of IGF-IR protein (Guvakova and Surmacz, 1997), ICI 182,780 moderately decreased IGF-IR levels in the presence of IGF-I. The action of ICI 182,780 and Tam on IGF-IR tyrosine phosphorylation was similar, namely, both compounds inhibited IGF-I-induced but not basal tyrosine phosphorylation of IGF-IR. The effects of ICI 182,780 and Tam on SHC were different. With Tam, we observed elevated tyrosine phosphorylation of SHC proteins and increased SHC/GRB2 binding in growth-arrested cells, while ICI 182,780 did not affect SHC phosphorylation or expression (Guvakova and Surmacz, 1997). Thus, induction of non-mitogenic SHC signaling is a peculiarity of Tam but not a ICI 182,780 mechanism of action.

In summary, cytostatic effects of ICI 182,780, similar to Tam, are associated with the inhibition of IGF-IR signaling. The mitogenic/survival IRS-1 pathway is a target for both antiestrogens. Both drugs reduce the levels of tyrosine phosphorylated IRS-1, but only ICI 182,780 clearly inhibits expression of the substrate. High cellular levels of IRS-1 hinder the response to higher doses of ICI 182,780, thus overexpression of IRS-1 in breast tumors may represent an important mechanism of antiestrogen resistance.

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Role of IRS-1 Signaling in Insulin-Induced Modulation of Estrogen Receptors in Breast Cancer Cells

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Cross-talk between steroid hormones and polypeptide growth factors regulates the growth of hormone-responsive breast cancer cells. For example, in the MCF-7 human breast cancer cell line, insulin up-regulates estrogen receptor (ER) content and binding capacity. Since the insulin receptor (IR) substrate 1 (IRS-1) is one of the core signaling elements transmitting mitogenic and metabolic effects of insulin, we investigated whether IRS-1 is also required for the insulin-induced function of the ER. The effects of insulin on the ER were compared in MCF-7 cells and MCF-7-derived cell lines with decreased levels (by ~80%) of IRS-1 due to the expression of IRS-1 antisense RNA. The severe IRS-1 deficiency in MCF-7 cells was associated with (1) reduced mitogenic response to 20 ng/ml insulin and 10% calf serum (CS), but not to 1 nM estradiol (E2); (2) loss of insulin-E2 synergism; (3) up-regulation of ER protein expression and binding capacity; and (4) loss of insulin-induced regulation of ER tyrosine phosphorylation. In conclusion, the data confirm the existence of the IR–ER cross-talk and suggest that IRS-1-dependent signaling may contribute to the negative regulation of the ER expression and function in MCF-7 cells.

The growth of hormone-responsive breast cancer cells *in vitro* and *in vivo* is controlled by steroids and polypeptide growth factors (1, 2). Accumulating evidence indicates that this growth control involves complex interactions, or cross-talk, between the two mitogenic systems. For instance, E2 stimulates the expression of several growth factors [such as insulin-like growth factors (IGFs), transforming growth factor alpha and beta, and amphiregulin], alters the levels or activity of different growth factor receptors [such as IGF-I and IGF-II receptors, and the epidermal growth factor (EGF) receptor], as well as modulates the expression of different IGF binding proteins (1–4). E2 is also able to modulate intracellular growth factor signaling pathways. For example, one of the acute effects of E2 binding to the ER in MCF-7 cells is stimulation of c-src tyrosine kinase and activation of c-src-dependent signaling substrates, including SHC (src-homology/collagen protein), which in consequence induces classic growth factor-responsive Ras/MAP (mitogen-activated kinase) cascade of kinases (5, 6). In addition, antiestrogens can block mitogenic action of growth factors on breast cancer cells through an ER-dependent or -independent mechanism (1–4, 7).

The other significant element of the cross-talk is modulation of ER expression and function by polypeptide growth factors (8–10). For example, different peptide mitogens can stimulate ER transcriptional activity even in the absence of E2 (11–17), probably through the phosphorylation of the ER on critical residues of Ser 118 and Tyr 537 (10, 16, 18). We have previously demonstrated that in MCF-7 cells, insulin up-regulates ER content and ER binding capacity, which is blocked in the presence of tyrosine kinase inhibitor, genistein (9). The intracellular signaling mechanism by which insulin regulates ER in breast cancer cells is not known. One of the major signaling pathways of the IR involves a substrate, IRS-1 (19). IRS-1 is a docking protein which becomes phosphorylated on multiple tyrosine residues immediately upon insulin binding to the IR. Tyrosine phosphorylated IRS-1 associates with different SH2-domain containing proteins activating a spectrum of downstream signaling pathways, such as Ras/MAP or PI-3 kinase pathways (19). The critical role of IRS-1 signaling in metabolic and mitogenic action of insulin has been well established in many cellular systems (19–21). In MCF-7 cells, IRS-1 is re-

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quired for monolayer and anchorage-independent growth, and is critical for transmitting signals controlling cell survival (21, 22). In this study, we examined the role of IRS-1 in IR-ER cross-talk.

MATERIALS AND METHODS

Cell lines. MCF-7/anti-IRS-1 clones 2 and 9 have been generated by stable transfection of MCF-7 cells with an expression plasmid encoding antisense IRS-1 RNA. The IRS-1 protein levels in MCF-7/anti-IRS-1 clones 2 and 9 have been down-regulated by approximately 80% and 85%, respectively. The other characteristics of these cells have been previously described in Ref. 22. In all experiments, the parental MCF-7 cells were used as a control. The clone overexpressing IRS-1 (MCF-7/IRS-1, clone 3) was obtained by stable transfection of MCF-7 cells with the CMV-IRS-1 plasmid that contains a mouse IRS-1 cDNA cloned into the Hind III site of the pRC/CMV mammalian expression vector (Invitrogen) (21). The resulting plasmid also confers neomicin resistance (inherent in pRC/CMV).

Routine cell culture. MCF-7 cells were grown in DMEM:F12 supplemented with 5% calf serum (CS); MCF-7/anti-IRS-1 cells were cultured in DMEM:F12 plus 5% CS plus 200 mg/ml G418 (22).

Experimental culture conditions. The cells cultured in growth medium were trypsinized and plated in phenol red-free (PRF) DMEM:F12 supplemented with 5% dextran-coated charcoal stripped CS (DCC-CS medium). After 24 h, this medium was changed to PRF-DMEM:F12 for another 24 h. Next, the cells synchronized in PRF-DMEM:F12 (day 0) were treated for 1, 2, or 96 h with PRF-DMEM:F12 containing 10% of dithiotreitol treated DCC-CS (DCC-SH-CS medium) supplemented with 20 ng/ml of insulin or 1 nM E2. DCC-CS and DCC-SH-CS were prepared as previously described (23, 24).

DNA content. Cells were plated in 24-well plates at a density of 1.5×10^4 cells/cm^2 in DCC-CS medium and then shifted to PRF-DMEM:F12 and DCC-SH-CS, as described above. At different times of treatment, cellular DNA content per well was assessed by fluorescent staining with Hoechst 33258, as described previously (9).

Estrogen receptor binding assay. ER binding sites in cytosol and nuclear fractions were determined by Scatchard analysis with ^{3}H-E2, as previously described (9). In brief, the cells were seeded at a density of 1.13×10^4/cm^2 in 100-mm culture plates in growth medium, then shifted to DCC-CS, PRF-DMEM:F12 and next to DCC-SH-CS, as described above. The synchronized cells were treated with mitogens for different times. Cytosol fractions were obtained by harvesting and sonicating the cells in a cytosol buffer (9). The sonicate was sedimented at 15,000 g for 30 min at 4°C. The supernatant contained the cytosol fraction, whereas the pellet contained cell nuclei.

Cytosol ER content. 50 ml of the cytosol fraction were incubated for 18 h at 4°C with 0.125–4 nM ^{3}H-E2. Non-specific binding was determined by incubating the cells with ^{3}H-E2 in the presence of 500-fold molar excess of diethylstilbestrol (DES). Bound and free E2 were separated by absorbing free hormones on DCC (100 μl) at 4°C for 15 min. The radioactivity of the bound hormone was determined in a scintillator counter.

Nuclear ER content. The nuclear pellet (obtained as described above) was resuspended in 0.6 M KCl for 1 h at 4°C, and then centrifuged at 15,000 g for 30 min at 4°C. Fifty microliters of the supernatant (nuclear fraction) was incubated with 2–64 nM of ^{3}H-E2 for 18 h at 4°C. The non-specific binding was determined with a 250-fold molar excess of DES.

Western blotting and immunoprecipitation. The protein levels and tyrosine phosphorylation of the ER were measured by immunoprecipitation (IP) followed by Western Blotting (WB), as previously described (7, 9). Following treatment, the cells were lysed in ice-cold lysis buffer (9). ER protein levels were determined by IP 500 μg of protein lysate with an anti-ER 304 antibody (Ab) (Neo Marker, Freemont USA), followed by WB using an anti-ER 311 Ab (Neo Marker, Freemont USA). CHO (ER-negative) cells were used as a negative control. Tyrosine phosphorylation of the ER was detected by immunoprecipitating 500 μg of protein lysate with an anti-ER 304 Ab (Neo Marker, Freemont USA), followed by immunoblotting with an anti-phosphotyrosine monoclonal Ab (mAb) PY20 (Santa Cruz Biotechnology, CA).

Statistical analysis. Data were analyzed using analysis of variance (ANOVA) or paired t-test, where applicable.

RESULTS

Down-regulation of IRS-1 inhibits growth-promoting effects of serum and insulin but not that of E2. The requirement for IRS-1 in IR-induced effects on the ER was studied using two MCF-7/anti-IRS-1 clones (2 and 9), in which the levels of IRS-1 were down-regulated by 80 and 85%, respectively. First, we investigated how decreased levels of IRS-1 affect growth response (measured as an increase of DNA content) to different mitogens. Figures 1A and 1B illustrates growth-promoting effects of 10% CS and 20 ng/ml insulin in MCF-7 and MCF-7/anti-IRS-1 cells synchronized in PRF-DMEM:F12. The mitogenic response to 10% CS in MCF-7/anti-IRS-1 clone 2 and 9, was suppressed by 55 (±SE 0.0) % and 57(±SE 0.0)%, respectively, compared that in the parental cells (Fig. 1A). Importantly, 20 ng/ml insulin did not produce any significant increase in DNA content in both MCF-7/anti-IRS-1 clones, whereas it stimulated DNA synthesis in the parental cells [40 (±SE 9.0)%; $p < 0.05$; Fig. 1B].

In contrast, the growth-promoting effect of 1 nM E2 in all cell lines was similar (Fig. 1B; variation among the cell lines: p = NS). In particular, in MCF-7 cells, E2 stimulation resulted in a 55 (±SE 11.1)% augmentation in DNA content over the basal level, and in MCF-7/anti-IRS-1, clones 2 and 9, the increase was 56 (±SE 15.3)% and 56 (± SE 11.2)%, respectively (Fig. 1B).

The stimulation of DNA synthesis by a combination E2 plus insulin was synergistic in all cell lines, but quantitatively smaller in MCF-7/anti-IRS-1 cells ($p < 0.05$, Fig. 1B) Specifically, whereas under the treatment, in MCF-7 cells DNA content increased 187 (±SE 40.0)%, in MCF-7/anti-IRS-1 clone 2 and 9, the stimulation was 142 (±SE 39.0)% and 88 (±SE 18.2)%, respectively.

Down-regulation of IRS-1 in MCF-7 cells is accompanied by the increase in both ER protein levels and ER binding capacity. We tested ER protein expression and binding capacity in MCF-7/anti-IRS-1 cells and, for comparison, in MCF-7/IRS-1 clone 3. All these cell lines express a neomycin resistance gene thus allowing us to test the eventual interfering effect of this gene on the ER. The basal content of the ER was clearly enhanced in both MCF-7/anti-IRS-1 clones with respect to MCF-7 cells. Interestingly, a 9-fold overexpression of IRS-1 in MCF-7/IRS-1, clone 3 did not significantly modulate ER expression (Figs. 2A and 2B). Scatchard

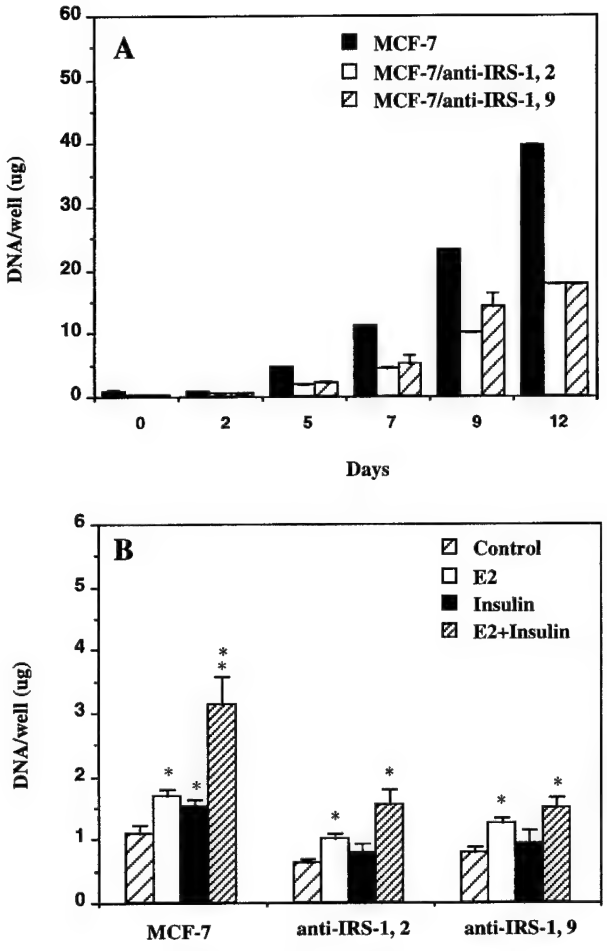

FIG. 1. Mitogenic effects of (A) 10% CS on MCF-7 and (B) Estradiol (E2), insulin or a combination of E2 and insulin on MCF-7 and MCF-7/anti-IRS-1 cells. The cells were synchronized in DCC-SH-CS medium (Day 0) and cultured in the presence of either 10% CS, 1 nM E2, 20 ng/ml insulin or 1 nM E2 plus 20 ng/ml insulin for 2–12 days. Cell DNA content was determined as described in Materials and Methods. *$p < 0.05$; **$p < 0.01$ versus control (DNA content at day 0).

analysis of E2 binding sites upon insulin stimulation confirmed significant upregulation of ER content in MCF-7/anti-IRS-1 clones compared with MCF-7 and MCF-7/IRS-1 cells (Fig. 3).

IRS-1 levels impact the regulation of basal and insulin-induced tyrosine phosphorylation of the ER. The regulation of the ER protein expression and tyrosine phosphorylation by insulin and E2 was determined in MCF-7 cells and in MCF-7/anti-IRS-1, clone 2 (Fig. 4). In the parental cells, the expression of the ER was elevated with both mitogens at 12 h and 96 h of treatment. In contrast, MCF-7/anti-IRS-1 cells expressed high basal levels of the ER that appeared refractory to insulin regulation, and were reduced by E2.

The basal ER tyrosine phosphorylation at 12 h was significantly elevated in MCF-7/anti-IRS-1 cells, compared with that seen in MCF-7 cells. Moreover, whereas in MCF-7 cells, insulin and E2 up-regulated ER phosphorylation, in MCF-7/anti-IRS-1 cells, these treatments had no effect on the ER phosphorylation status (Fig. 4B). At 96 h, basal ER tyrosine phosphorylation was similar in both cells lines, but it was clearly down-regulated by insulin in MCF-7 cells and not affected in MCF-7/anti-IRS-1 cells. E2 at 96 h induced the phosphorylation of the ER in both cell lines.

DISCUSSION

Cross-talk between signaling pathways of the IR or the IGF-IR and the ER is a powerful mechanism controlling the growth of many hormone-responsive breast cancer cells. It is thought that deregulation of this cross-talk may lead to the development of hormone-independence and antiestrogen-resistance (1, 3, 4). The molecular basis of growth factor-steroid communication are not entirely clear. Although considerable knowledge exists about the effects of estrogens on IGF or insulin systems, the role of these growth factors on ER expression and function is less known. In this context, of particular interest are recent data demonstrating that IGF or insulin are able to up-regulate E2 binding sites and stimulate the transcription of E2-responsive DNA, even in the absence of E2 (8, 9, 11, 17). In the latter case, growth factors appear to induce phosphorylation of the unligated ER on Ser 118 (via MAP kinase pathway), and possibly on Tyr 537, in consequence enhancing ER transcriptional activity (10, 18, 25). These observations, together with our present results, provide the evidence that the IR modulates ER function on at least three different molecular levels: a) ER protein expression; b) ER binding capacity; and c) ER phosphorylation. The post-receptor events involved in the control of the ER by insulin are

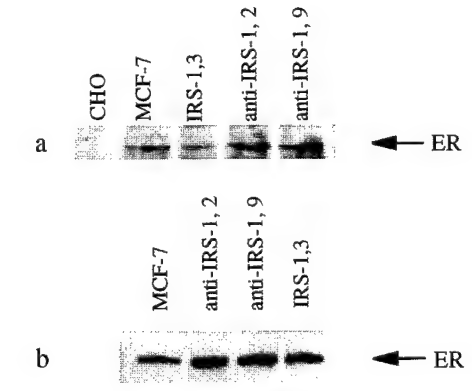

FIG. 2. ER protein content in growing MCF-7 cells, MCF-7/anti-IRS-1, clones 2 and 9, and in MCF-7/IRS-1, clone 3. ER protein content was determined in whole cell lysates (a) by WB or by IP followed by WB with specific anti-ER Ab (b) as described in Materials and Methods.

still poorly characterized. Here, we examined the role of IRS-1, a principal substrate of the IR that is critical for its metabolic and mitogenic action, in the modulation of ER expression and tyrosine phosphorylation.

First, IRS-1 was not critical for the stimulation of ER binding capacity by insulin, since in MCF-7 cells with significantly (~80%) decreased levels of IRS-1, insulin normally up-regulated E2 binding sites (Fig. 3), whereas its growth-promoting action was inhibited under the same conditions (Fig. 1B). The possibility that other IR-dependent signaling pathways, such as SHC or PI-3 kinase pathways, are responsible for stimulating E2 binding sites in MCF-7 cells is currently under investigation.

Second, IRS-1 signaling may contribute to a physiological down-regulation of ER protein levels in MCF-7 cells, as the reduction of IRS-1 levels was clearly paralleled by an increase of ER expression and binding capacity (Fig. 1B, 2, and 4). However, overexpression of IRS-1 did not reduce ER levels, which suggest that the regulation of the ER depends on some other signaling pathways. Why lower levels of IRS-1 trigger ER overexpression is not known. Perhaps, when IR-dependent mitogenicity is compromised, as occurred in MCF-7/anti-IRS-1 cells, a compensatory mechanism stimulates an overexpression of the ER. Interestingly, however, this ER overexpression in MCF-7/anti-IRS-1 clones was not reflected by an increased mitogenic response to E2 (Fig. 1B), indicating that stimulation of cell growth by E2 is a saturable process, possibly controlled by a negative effect of estradiol on its own receptor (Ref. 26 and Fig. 4A).

Third, our studies suggest that IRS-1 is important for the regulation of ER tyrosine phosphorylation, at least in cells exposed to insulin for 96h. Specifically, such a long-term insulin treatment evidently reduced the ER phosphorylation in the parental cells, but it produced no effect in MCF-7/anti-IRS-1 cells.

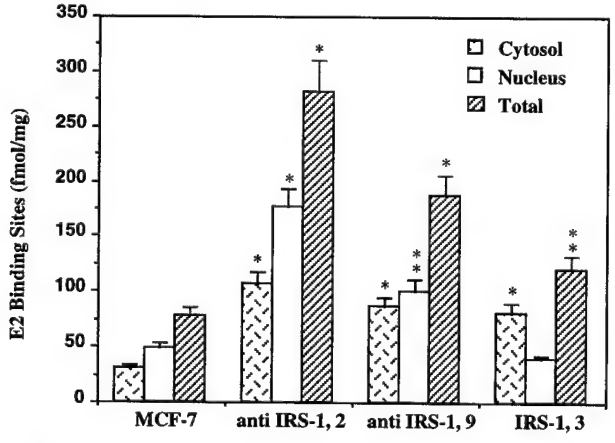

FIG. 3. Binding capacity of cytosolic, nuclear and total ER under basal conditions and upon insulin (20 ng/ml) stimulation in MCF-7 cells, MCF-7/anti-IRS-1 clones 2 and 9, and in MCF-7/IRS-1, clone 3. *$p < 0.05$; **$p < 0.01$ versus control.

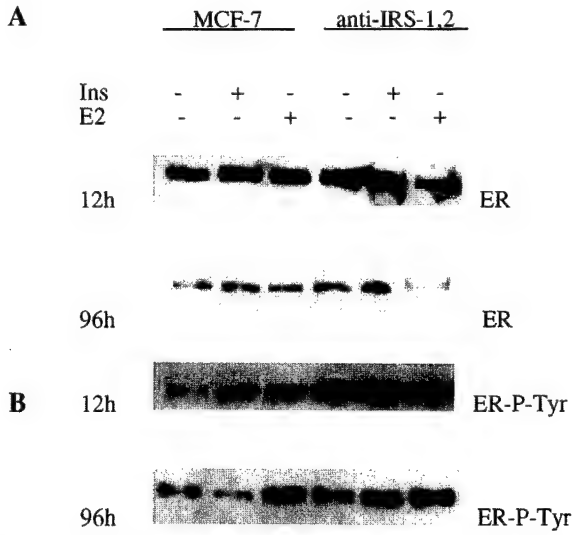

FIG. 4. (A) E2 protein content (ER) in MCF-7 and in MCF/anti-IRS-1, clone 2. The levels of ER in cells untreated or treated with either insulin (20 ng/ml) or estradiol (1 nM) were determined by IP and WB at 12 and 96 h. (B) Tyrosine phosphorylation of the ER (ER-P-Tyr) in MCF-7 and MCF-7/anti-IRS-1 clone 2 in cells untreated or treated with either insulin (20 ng/ml) or E2 (1 nM) for 12 or 96 h. ER content and ER tyrosine phosphorylation were determined by stripping the blots from Fig. 4A. and reprobing with an anti-phosphotyrosine mAb as described under Materials and Methods.

The biological relevance of ER tyrosine phosphorylation is still under debate. It is possible that overall tyrosine phosphorylation of the ER is not directly related to E2 transcriptional and growth effects, as already suggested by other investigators; for example ER phosphorylation has been shown to be induced by both estrogen and antiestrogens (11). The concept that in our system, impaired IRS-1 signaling affected phosphorylation of the ER on Ser 118, in consequence reducing ER transcriptional activity is currently under investigation.

In summary, IRS-1 pathway appears to be required for IR-dependent proliferation in MCF-7 cells, but not for E2-stimulated growth. In addition, the data suggest that IRS-1 may contribute to the process of physiologic downregulation of ER expression and function.

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IGF-IR Stimulates Breast Epithelial Cell Motility via Reorganization of the Actin Cytoskeleton, Remodeling of Focal Contacts, and Modulation of the Phosphorylation Status of Focal Adhesion Proteins: FAK, Cas and Paxillin. M. A. Guvakova, E. Surmacz.
Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107.

Insulin-like growth factor (IGF-I) is known to promote the motility of different cell types. We investigated the role of IGF-I receptor (IGF-IR) signaling in motility of MCF-7 breast epithelial cells overexpressing the wild type IGF-IR. The elevated level of the IGF-IR correlated with augmented migration of cells in vitro. The treatment of subconfluent serum-starved cells with 50 ng/ml IGF-I induced rapid morphological transition towards mesenchymal phenotype and resulted in disruption of polarized cell monolayer. Immunofluorescence staining of IGF-I-treated cells with rhodamin-phalloidin revealed dynamic changes in the actin cytoskeleton marked by disassembly of long actin fibers within 5-15 min, followed by development of meshwork of short actin bundles localized to multiple membrane protrusions. In parallel, IGF-I induced transient dephosphorylation of focal adhesion-associated proteins: p125 focal adhesion kinase (FAK), p130 Crk-associated substrate (Cas) and paxillin. Pretreatment of cells with 5 μM phenylarsine oxide (PAO), an inhibitor of phosphotyrosine phosphatases, rescued FAK and its associated proteins Cas and paxillin from IGF-I-induced tyrosine dephosphorylation. PAO-pretreated cells were refractory to morphological transition and did not develop cellular protrusion in response to IGF-I. Additionally, PAO inhibited migration of MCF-7/IGF-IR cells in a time and concentration dependent manner. Our results suggest that in MCF-7 cells stimulation of the IGF-IR activates a putative tyrosine phosphatase acting upon focal adhesion proteins and promoting the reorganization of focal contacts. This process is associated with dynamic remodeling of the actin cytoskeleton. Coordinated regulation of these events is required for induction of MCF-7 cell motility.

173 ROLE OF SHC SIGNALING IN BREAST CANCER CELL ADHESION AND MOTILITY. Mauro, L., Sisci, D., Salerno, M., Ando, S., and Surmacz, E. Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pa 19107 and Department of Cellular Biology, University of Calabria, Italy.

SHC proteins (p47, p52, and p66) are signaling substrates for most receptor and cytoplasmic tyrosine kinases. Tyrosine phosphorylation of SHC leads to GRB2/SOS binding and induction of Ras/MAP signaling cascade. An important role of SHC in cell proliferation and transformation has been well established. In addition, recent data demonstrated that SHC associates with certain integrins and participates in integrin signaling.

In many breast cancer cell lines, the levels of SHC are higher compared with that in normal epithelial cells, however, the status of SHC in breast tumors has not been studied. To study the role of SHC in the biology of breast cancer cells, we developed several MCF-7-derived cell lines overexpressing this substrate (MCF-7/SHC cells). In MCF-7/SHC cells, an 8-fold overexpression of SHC brought about a moderately increased responsiveness to IGF-I and EGF (20-70% increase over that in MCF-7 cells), and altered dynamics of growth factor-induced MAP kinase activity. SHC overexpression did not improve the ability of cells to grow under anchorage-independent conditions.

In MCF-7/SHC cells, the amount of SHC associated with alpha 5 beta 1 integrin, a fibronectin (FN) receptor, was ~ 6-fold greater than that in the parental cells or in other 4 cell lines (overexpressing the IGF-IR or its substrate IRS-1). When plated on FN, MCF-7/SHC cells attached faster than other tested cell lines (1 h vs. 2-3 h). This fast attachment was accompanied by earlier decline of adhesion-induced MAP kinase activity. The attachment of cells to FN was associated with decreased binding of p47 SHC to alpha 5 beta 1 integrin. Conversely, stimulation with EGF caused detachment of cells from FN, and increased binding of p47 to alpha 5 beta 1 integrin. The motility of MCF-7/SHC cells tested in FN-coated inserts was inhibited compared with that of the other cell lines. However, in the presence of EGF or IGF, the motility of SHC overexpressing cells was greatly (up to 300 %) increased, whereas it was only minimally altered in other cell lines (from -20 to + 40%). These data suggest that in breast cancer cells, SHC transmits not only growth promoting signals, but is also involved in dynamic regulation of cell adhesion and motility

174 **THE AGGRESSIVITY OF BREAST CANCER CELLS IS DECREASED BY THE OVEREXPRESSION OF THE CYTOSKELETAL PROTEIN KERATIN 18 (K18).** Buehler H, Kuhle A, Bangemann N, Becker C and Schaller G, Dept. of Gynecology and Obstet., MC Benjamin Franklin, Freie Universität, 12200 Berlin, Germany

The decisive factor in prognosis of human breast cancer is the metastasis of the primary tumor. In a retrospective study we could show that high expression of K18 in the cancer cells is correlated with a favorable prognosis of the patient. To get insight into the underlying mechanisms we have transfected the K18 gene into a low expressing highly malignant breast cancer cell line.

The experiments were performed with the MCF-7 derivative LCC2. This metastatic and invasive subclone is characterized by a low K18 expression in contrast to the much less aggressive parental cells which show high levels of K18. The complete human K18 gene (pGC 1853, R. Oshima La Jolla, CA) was transfected by using PerFect Lipid pFx-2 (Invitrogen).

(i) The adhesivity of the transfected cells is strongly increased. Freshly trypsinized cells were agitated on an orbital shaker and the cell aggregation was observed. Free single cells decreased more rapidly and the aggregates formed were significantly larger for the K18-cells. (ii) The ability of the cells to grow anchorless in soft agar was dramatically altered. Within 10 days the controls formed big colonies whereas the K18-cells were only able to divide for a few cycles followed by rapid apoptosis.

The intermediate filaments in epithelial cells are formed by cytokeratins and K18 is a marker of well differentiated luminal cells in the breast epithelial tissue. The metastatic event as a rule is based on cellular dedifferentiation with changes in the cytoskeletal structure. Thereby keratins are very often replaced by vimentin. The expression of K18 seems to hamper the metastatic process. The reversal of this dedifferentiation by transferring the K18 gene into the cells obviously results in a reduced aggressivity of the cancer cells.

175 **DIFFERENT EXPRESSION PATTERNS OF MMP-2 AND MMP-9 IN BREAST CANCER.** Rha SY, Shim KY, Ahn JB, Gong SJ, Yoo NC, Yang WI, Kim JH, Roh JK, Min JS, Lee KS, Kim BS and Chung HC. Yonsei Cancer Research Institute, Yonsei Cancer Center, Department of Internal Medicine, Pathology, General Surgery, Yonsei University College of Medicine, Seoul, Korea

Among the many biological characteristics of cancer, the matrix metalloproteinase(MMPs) is essential for tumor invasion and metastasis. The relationship between MMP-2 and MMP-9 according to tumor progression has not been studied yet. We evaluated the synchronous expression and activation rate of MMP-2 and MMP-9 in breast cancer tissues and compared them to the clinical parameters in order to determine the clinical significance of MMPs and the possibilities of using them as a therapeutic target.

The activity of MMPs was evaluated in 121 breast cancer tissues using zymography and the area of activation was calculated by computer-assisted densitometry in comparison to the activity of a positive control(HT-1080). In 121 tumor tissues, 32(26.4%) didn't express any form of MMPs and 19(15.7%) showed both expression of MMP-2 and MMP-9. We observed that only one tissue expressed MMP-9 alone, while MMP-2 alone was expressed in 69 tissues. In 88 patients with MMP-2 and/or MMP-9 expression, we were unable to observe any correlation between the activity of MMPs expression or activation rate and the clinical parameters. But MMP-2 and MMP-9 activity increased according to T factor. Rapid production of MMP-9 occurred from T2(p=0.046), while that of MMP-2 occurred from T3(p=0.004)

In conclusion, MMPs activity was organ specific. The major MMPs in breast cancer was MMP-2 and MMPs activity was abst.docdifferent with tumor progression. When we try to use MMPs as a specific therapeutic target, we should use different inhibitors according to tumor size in patients at the same stage.

176 **LOSS OF HETEROZYGOSITY AT D14S62 IS INVERSELY CORRELATED WITH METASTATIC POTENTIAL**

O'Connell P[1], Fischbach K[1]*, Hilsenbeck S[2], Fuqua SAW[2], Mohsin SK[1],Martin M[1], Clark GM[2], Osborne CK[2], and Allred DC[1].

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In studies of human cancer evolution, the prevalence of loss of heterozygosity (LOH) is generally proportional to the stage of the lesion under study, increasing in frequency from non-invasive to invasive to metastatic cancers. In this study, high rates of LOH were observed at marker D14S302 in approximately 60% of primary breast tumors, indicating a potential tumor suppressor gene in this vicinity. Unexpectedly, more distal portions of chromosome 14q (near markers D14S62, and -S51) showed high rates of LOH in node-negative (D14S62, 59%; D14S51, 40%) but not in node positive (D14S62, 18%: D14S51, 13%) primary breast tumors. Our hypothesis is that a metastasis-related gene resides in the D14S62 region and a deletion of one copy of this gene may slow down the metastatic spread of breast cancer.

Analysis of interstitially-deleted cases of node negative samples place the putative metastatic-related gene within an approximately 1460kb region. We have assembled a yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) contigs across this region. Positional candidate cloning (mapping of expressed sequence tagged cDNA clones to our YAC and BAC contigs) and direct cDNA selection (isolation of novel cDNAs by solution hybridization selection with our BAC clones) techniques have identified 20 candidate cDNAs to date. Fragments of these cDNA's have been cloned into the pcDNA vector (Invitrogen) in an antisense orientation and stably transfected into the human metastatic breast cell line MDA-MB-435. We are in the process of choosing cells which express high levels of the antisense transcripts for injection into mammary fat pads of female athymic mice. This in vivo functional assay will identify putative metastatic-related gene(s) by inhibition of MDA-MB-435 breast cancer cell metastasis to lung.

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Tyrosine kinase activity of the IGF-IR is required for the development of breast cancer cell aggregates in three-dimensional culture. Guvakova, M. A., Surmacz, E. *Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107*

In human breast carcinomas, the expression and tyrosine kinase activity of the insulin-like growth factor type I receptor (IGF-IR) is increased relative to normal breast epithelium. However, the consequences of such IGF-IR amplification in the development and progression of breast cancer are yet to be fully defined. To further explore the functional role of the IGF-IR kinase in breast cancer biology, we developed stable clones of MCF-7 breast cancer cells overexpressing kinase-deficient mutants of the human IGF-IR. IGF-IR-mediated signaling and three-dimensional (3-D) cell growth on the extracellular matrix (ECM) were investigated using the clones with the highest levels of mutant IGF-IRs. The overexpression of the IGF-IR/YF3 mutant, containing a triple tyrosine mutation in the kinase domain, abolished several IGF-I-dependent effects, including tyrosine phosphorylation of the IGF-IR and its substrates IRS-1 and SHC, formation of downstream signaling complexes of IRS-1/PI-3-kinase and SHC/Grb-2, and activation of MAP kinases (ERK1/ERK2). The overexpression of the IGF-IR/KR, with an inactive tyrosine kinase ATP-binding site, abrogated SHC signaling, reduced IGF-IR and IRS-1 tyrosine phosphorylation, and attenuated MAP kinase activity. In 3-D culture, the clones expressing the dominant-negative IGF-IR mutants demonstrated significantly reduced compared to MCF-7 cells ability to form large multicellular aggregates (≥300 μm): 57% and 97% inhibition in cells overexpressing IGF-IR/YF3 and IGF-IR/KR, respectively. The kinase-deficient IGF-IR mutants, however, retained the ability to localize at cell-cell contacts and associate with adherence junction proteins E-cadherin, α- and β-catenins. We conclude that IGF-IR tyrosine kinase activity is required for the development of the large aggregates of breast tumor cells growing on ECM in 3-D culture. (Supported by DAMD 17-97-1-7211 and NIH DK 48969)

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IGF-IR Tyrosine Kinase is Required for Breast Cancer Epithelial Cell Motility. Marina A. Guvakova and Ewa Surmacz. Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107, USA.

Insulin-like growth factor (IGF-I) is known to promote the motility of different cell types. We investigated the role of IGF-I receptor (IGF-IR) signaling in motility of MCF-7 breast epithelial cells over-expressing either the wild type or kinase-inactivated mutants of the IGF-IR. The elevated level of the wild type IGF-IR correlated with augmented migration of cells in vitro, whereas migration was not increased in cells over-expressing the similar level of the catalytically inactive IGF-IR. IGF-I stimulation of subconfluent wild type IGF-IR over-expressing cells induced rapid morphological transition towards mesenchymal phenotype and resulted in disruption of polarized cell monolayer. Immunofluorescence staining of IGF-I-treated cells with rhodamine-phalloidin revealed dynamic changes in the actin cytoskeleton marked by disassembly of long actin fibers within 5 min, followed by development of meshwork of short actin bundles localized to multiple membrane protrusions. In parallel, IGF-I induced transient dephosphorylation of focal adhesion-associated proteins: p125 focal adhesion kinase (FAK), p130 Crk-associated substrate (Cas) and paxillin. Pretreatment of cells with 5 μM phenylarsine oxide (PAO), an inhibitor of phosphotyrosine phosphatases, rescued FAK and its associated proteins Cas and paxillin from IGF-I-induced tyrosine dephosphorylation. PAO also inhibited development of IGF-I-induced membrane protrusions and blocked cell migration. Our results suggest that IGF-IR tyrosine kinase directly or indirectly activates a putative tyrosine phosphatase acting upon focal adhesion proteins. IGF-IR signaling is required for dynamic remodeling of the actin cytoskeleton and focal adhesion contacts during IGF-I-induced motility in MCF-7 cells. (DAMD 17-97-1-7211, NIH DK 48969)

Function of the IGF-I Receptor in Breast Cancer

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Abstract

The insulin-like growth factor-I receptor (IGF-IR) is a transmembrane tyrosine kinase regulating various biological processes such as proliferation, survival, transformation, differentiation, cell-cell and cell-substrate interactions. Different signaling pathways may underline these pleiotropic effects. The engagement of specific pathways depends on the number of activated IGF-IRs, availability of intracellular signal transducers, action of negative regulators, and is influenced by extracellular modulators.

Experimental and clinical data implicate the IGF-IR in breast cancer etiology. There is strong evidence linking hyperactivation of the IGF-IR with the early stages of breast cancer. In primary breast tumors, the IGF-IR is overexpressed and hyperphosphorylated, which is associated with radio-resistance and tumor recurrence. In vitro, the IGF-IR is often required for mitogenesis and transformation, and its overexpression or activation counteracts effects of various pro-apoptotic treatments.

In hormone-responsive breast cancer cells, IGF-IR function is strongly linked with estrogen receptor (ER) action. The IGF-IR and the ER are co-expressed in breast tumors. Moreover, estrogens stimulate the expression of the IGF-IR and its major signaling substrate IRS-1, while antiestrogens downregulate IGF-IR signaling, mainly by decreasing IRS-1 expression and function. On the other hand, overexpression of IRS-1 promotes estrogen-independence for growth and transformation.

In ER-negative breast cancer cells, usually displaying a more aggressive phenotype, the levels of the IGF-IR and IRS-1 are often low and IGF is not mitogenic, yet the IGF-IR is still required for the metastatic spread. Consequently, IGF-IR function in the late stages of breast cancer remains one of the most important questions to be addressed before rational anti-IGF-IR therapies are developed.

Key words: breast cancer, insulin-like growth factor I receptor, IRS-1, estrogen-independence, antiestrogens, metastasis

The insulin-like growth factors I and II (IGFs) act as endocrine, paracrine or autocrine regulators of various biological processes in normal and neoplastic cells. The actions of IGF-I in the adult are mediated primarily by the type I insulin-like growth factor receptor (IGF-IR), while IGF-II stimulates both the IGF-IR and the insulin receptor (IR) (1, 2). It has been well established that in many cell types, activation of the IGF-IR is essential for cell survival, transformation, and hormone-independence--the processes that promote tumorigenesis (3-6). During the past several years, the impact of the IGF-IR on breast cancer development and progression has also been recognized, providing a new direction in designing anti-growth factor compounds for breast cancer therapy.

IGF-IR expression and structure. Almost all cell types, except hepatocytes and T-lymphocytes, express the IGF-IR (1, 3). The IGF-IR is encoded by a 100 kb gene containing 21 exons located on the distal arm of chromosome 15 (1, 7). The IGF-IR promoter region is GC-rich, similar to other housekeeping genes lacks TATA or CCAAT boxes, but contains several sites for binding transcriptional factors such as SP-1, E2F, and early growth response (EGR) proteins (5, 8, 9). The expression of the IGF-IR is regulated by different physiologic stimuli and may be altered in certain pathologies (e. g., diabetes, cancer). For instance, IGF-IR mRNA is enhanced by growth hormone, follicle stimulating hormone, luteinizing hormone, thyroid hormones, glucocorticoids, and estrogens (9). Moreover, different mitogens (e.g., platelet-derived growth factor, fibroblast growth factor) or oncoproteins (e.g., c-myb, hepatitis B Hbx) can induce IGF-IR transcription. Conversely, IGF-IR expression is downregulated by high concentrations of IGF-II,

interferon, antiestrogens, and tumor supressors (e.g. Wilms' tumor or p53 proteins) (4, 8, 9).

The major IGF-IR 11 kb transcript is translated into a single 1,367 amino acid (aa) (180 kDa) precursor protein, which is then cleaved to form 135 kDa alpha and 90 kDa beta subunits. A mature IGF-IR is a heterotetramer composed of two alpha and two beta subunits linked by disulfide bonds (Fig. 1). The extracellular alpha subunits are responsible for ligand binding. IGF-IR beta subunits, which contain short transmembrane and large intracellular segments, transmit ligand-induced signal (1, 7, 9). Within the beta subunit, three major domains have been recognized: a tyrosine kinase domain, a juxtamembrane part, and the C-terminus, each containing residues essential for different IGF-IR functions (Fig. 1). Specifically, in the kinase domain, the ATP binding site containing lysine (Lys) 1003 as well as the tyrosine (Tyr) cluster (Tyr 1131, 1135, 1136) are critical for the catalytic activity of the receptor (5, 9, 10). In the juxtamembrane domain, Tyr 950 flanked by the NPEY motif is required for recruiting major signaling substrates such as insulin receptor substrates (IRS) 1-4 and src/collagen-homology (SHC) proteins (5, 6, 9, 10). The C-terminus contains several residues essential for IGF-I signaling, including Tyr 1250, Tyr 1251, a stretch of serines (Ser) 1280-1283, histidine (His) 1293, Lys 1294, and Tyr 1316. In particular, the region between residues 1229 and 1245 has been found necessary for the association of an adapter GRB10, Tyr 1251 is required for binding a putative substrate p28, Ser 1280-1283 are necessary to sequester an adapter 14-3-3 epsilon, and Tyr 1316 is capable of recruiting either p85 subunit of phosphatidyl inositol-3 kinase (PI-3K) or SHPTP2 phosphatase (5, 9, 10, Baserga et al.

unpublished data). According to recent evidence, Tyr 1251 also appears to be indirectly involved in binding of SHC to the IGF-IR (11).

The IGF-IR shares a significant structural homology with the IR. The kinase domains of these receptors are 80-90% identical. Also, Tyr 950 of the IGF-IR has its equivalent, Tyr 960, in the juxtamembrane domain of the IR (7). Importantly, the C-terminal regions of the receptors are quite different, sharing only approximately 40% homology. The equivalents of Tyr 1250 and 1251, Ser 1280-1283, and aa 1293-1301 are not present in the IR. Consequently, it is believed that the differences between biological responses of the IGF-IR and IR are associated with the induction of specialized signaling pathways arising from the C-terminus (1, 7, 10).

IGF-IR signaling. Upon ligand binding, IGF-IRs clusterize and tyrosine kinase is activated leading to autophosphorylation and transphosphorylation of beta subunits (1). Phosphorylation of specific Tyr and Ser residues creates binding sites for IGF-IR signaling substrates. The best known substrates are docking proteins IRS-1 and SHC. Both bind Tyr 950 through their phosphotyrosine binding (PTB) domain (4, 9).

IRS-1 is a remarkable effector of the IGF-IR, capable of amplification and diversification of the signal because it can recruit various signaling molecules and induce numerous cellular responses. IRS-1 contains about 20 tyrosine phosphorylation sites which can directly bind signaling molecules equipped with tyrosine binding domains, such as src-homology 2 (SH2) domains. For instance, there are about nine YMXM motifs in IRS-1 that can attract p85 subunit of PI-3K via SH2-type interactions, and other domains

recruiting SH2-containing adapters GRB2, Nck, and Crk, SHP2 phosphotyrosine phosphatase, and Fyn kinase. Other partners of IRS-1, such as integrin alpha v beta 3 or the adapter 14-3-3, associate with the substrate through unknown mechanisms (12).

The major pathway induced by tyrosine phosphorylation of IRS-1 is PI-3K, whose downstream effectors are Ser/threonine kinases Akt, $p70^{S6}$, and some isoforms of protein kinase C (PKC) (13). PI-3K is involved in the regulation of mitogenesis, metabolism, and actin cytoskeleton rearrangements, and PI-3K/Akt pathway has recently been recognized as one of the most important signals ensuring cell survival. One of the cellular targets of Akt is a pro-apoptotic protein BAD which induces cell death when bound to anti-apoptotic proteins Bcl2 or Bcl_{XL}. The phosphorylation of BAD by Akt facilitates its sequestration by 14-3-3 adapters and prevents apoptosis. Another effector of Akt is $p70^{S6}$ kinase which activates expression of cyclin D1 initiating cell cycle progression (13, 14).

Association between IRS-1 and GRB2/SOS complex leads to the stimulation of the classic Ras/MAP cascade of kinases, the pathway that is implicated in a broad array of biological responses, including cell growth and differentiation (12, 15). Ras/MAP pathway can also be induces through Nck or Crk adapters binding to IRS-1 or by GRB2 binding to IGF-IR-associated SHC proteins (6, 12, 15).

Three additional IRS proteins (IRS-2, -3, and -4) exhibiting different degrees of structural homology to IRS-1 have been cloned. The activities of IRS-1 and IRS-2 appear to partially overlap, for instance, in IRS-1-knock-out mice, IRS-2 substituted IRS-1 function in stimulating PI-3K and activating glucose metabolism. However, in IRS-1-

deficient fibroblasts, only IRS-1, but not IRS-2, reconstituted cell cycle progression (12, 13). The functions of IRS-3 and -4 are not well understood. Similarly, the pathways initiated by the binding of GRB10 or 14-3-3 to the IGF-IR are still obscure.

IGF-IR signal specificity. Because the IGF-IR may regulate many, often contradictory (growth vs. differentiation), processes, it is of great importance to understand how signal specificity is achieved. Here, I will focus on IGF-IR-dependent survival, mitogenesis, and anchorage-independent growth, the most studied IGF effects. The current view is that IGF-I response is dictated by the engagement of different sets of intracellular pathways. Which pathways are stimulated depends on (i) the number of activated receptors on the cell surface; (ii) the availability of signaling substrates and receptor-substrate binding sites; (iii) the abundance and activity of negative regulators such as phosphatases; and (iv) extracellular context, e.g., ligand availability or extracellular matrix (ECM) components and their interaction with cells.

The first point is best illustrated by the work of Rubini et al. and Reiss et al., who analyzed the relationship between the number of IGF-IR expressed on the cell and IGF-I-induced biological response (16, 17). While activation of $2\text{-}10\text{x}10^3$ receptors stimulates tyrosine phosphorylation of IRS-1 as well as activation of an early response gene c-myc, it is not sufficient for SHC phosphorylation or the entry of cells into the cell cycle. With $1{,}5\text{x}10^4$ receptors, the cells progress through the S phase, but they are not able to complete cell division, and their survival ability under anchorage-independence is minimal. The increase of receptor expression to $2{,}2\text{-}6{,}0\text{x}10^4$ receptors/cell ensures phosphorylation

of the major substrates, full mitogenic response, and good survival, but produces only a weak transforming activity (measured by growth in soft agar). Activation of more than $1x10^5$ IGF-IRs provides signal strong enough to activate both IRS-1 and SHC signaling pathways, stimulate cell division, and support robust transformation (16, 17). A direct relationship between the number of stimulated IGF-IRs and cell survival and/or tumorigenesis in animal models has been documented by Baserga and LeRoith laboratories (18, 19). For instance, NIH 3T3 mouse fibroblasts expressing $1.9x10^5$ IGF-IR/cell form tumors in nude mice, while fibroblasts with lower IGF-IR levels ($1.6x10^4$) are non-tumorigenic. In addition, the latency of tumor formation in vivo was reduced with high doses (4-10 mg/kg) of endocrine IGF-I, suggesting that chronic stimulation of a high number of IGF-IRs was critical for the onset of tumorigenesis (19).

To determine whether various functions of the IGF-IR are induced by overlapping or distinct pathways, a mutational analysis has been performed. In the studies of Baserga et al., different mutant IGF-IRs have been expressed in R-minus cells (derived from IGF-IR knock-out mice), which allowed analyzing signaling pathways of the mutants without interference of the endogenous wild-type IGF-IRs (10). This work has been complemented by O'Connor et al. who studied mutant receptors expressed in either hematopoietic IRS-1-negative FL5.12 cells or apoptosis-prone Rat-1/Myc fibroblasts, and by LeRoith et al. who used NIH 3T3 fibroblasts for the analysis (5, 6). All studies demonstrated that a mutation in the ATP binding site produced "dead" receptors incapable of signal transmission. Replacement of all three Tyr 1131, 1135, and 1136, or Tyr 1136 alone, with phenylalanine produced a receptor that was not mitogenic or

transforming, but it was still inducing an efficient survival signal. Mutations in either Tyr 1131 or Tyr 1135 downregulated transformation without reducing cell growth. Tyr 950 in the IGF-IR juxtamembrane domain was found necessary for IRS and SHC association, and for induction of mitogenic and transforming activity. Interestingly, however, the IGF-IR/Tyr 950 mutant transmitted anti-apoptotic signaling. This indicates that in addition to the classic IRS-1-dependent PI-3K/Akt pathway, other survival pathway(s) emanates from the IGF-IR (5, 6, 9, 10).

Deletion of the entire C-terminus at aa 1229 produced a receptor that retained normal mitogenic function but was totally lacking transforming potential (20). Subsequent detailed studies with mutant IGF-IRs expressed in R-minus cells mapped the "transforming domain" between residues 1245 and 1310, with Tyr 1251, Ser 1280-1283, His 1293, and Lys 1294 required for transformation (10). Importantly, this region does not have an exact counterpart in the IR. Indeed, the expression of the IR or a chimeric IGF-IR containing an IR C-terminus did not support soft agar growth of R-minus cells (10). Notably, the IGF-IR transforming signal appears to be truly unique, at least in mouse fibroblasts, as overexpressing of various growth factor receptors, signaling molecules or oncogenes (except for v-src), singly or in combination, did not restore transformation in R-minus cells, while the IGF-IR did (3, 21). The mediators of the IGF-IR transforming pathway are not yet known, but the adapters 14-3-3 and GRB10, which bind to the C-terminus, could be involved.

Interestingly, the IGF-IR C-terminus also appears to play a unique role in survival signaling. Mutants with a deleted C-terminus (at residues 1229 or 1245) retained or even

amplified anti-apoptotic function, while single mutations in Tyr 1251, His 1293 and Lys 1294 reduced survival. Consequently, it has been suggested that the C-terminus is an intrinsic inhibitory domain of the IGF-IR, while the residues Tyr 1251, His 1293, Lys 1294 act as neutralizers of this pro-apoptotic function. Indeed, expression of the C-terminal 108 aa as a membrane-targeted protein resulted in induction of apoptosis, and mutations in Tyr 1250/1251 and His 1293/Lys 1294 abrogated this cytotoxic activity (5).

To summarize, IGF-IR signals required for mitogenesis, transformation, and survival are distinct but partially overlap. For instance, no transforming activity is seen in the absence of mitogenic activity. Transformation also seems to have some common pathways with IGF-dependent survival. However, cell survival can be induced by a weak signal which is not sufficient for mitogenesis or transformation, while transformation requires strong IGF-IR activation and induction of specific signals originating at the C-terminus.

The pathways mediating non-growth IGF-I responses such as cell-cell or cell-substrate interactions are less well characterized. Our preliminary data indicate that in epithelial cells, intercellular adhesion requires the tyrosine kinase domain as well as the C-terminus of the IGF-IR, and depends on SHC but not on IRS-1 signaling (4, 22, Surmacz et al., unpublished data). IGF-I-induced motility and reorganization of actin cytoskeleton involves PI-3K and SHC activities, and modification of proteins associated with focal adhesions (22, 23).

It is known that IGF-IR response may be cell-type specific (24). One mechanism ensuring such specificity is the availability of intracellular signaling intermediates. For

instance, with the same cellular content of the IGF-IR, downregulation of IRS-1 expression inhibits cell growth, transformation, and results in cell death, while amplification of IRS-1 sensitizes cells to low concentrations of IGF-I and enhances anchorage-independent growth (22, 25, 26). On the other hand, overexpression of SHC does not improve IGF-I-dependent growth, but inhibition of SHC expression inhibits cell growth, transformation, and to a lesser extent, cell survival (22, Surmacz et al., submitted).

Finally, the extracellular context plays a role in IGF-I response, for instance, survival and growth of cells adhering to a proper substrate is mediated through the IRS-1 pathway, while the same pathway is much less important in IGF-I-dependent protection from apoptosis in anchorage-independence (22, 27, 28).

Requirement for IGF-IR in proliferation, transformation and survival of breast cancer cells. The critical role of the IGF-IR in breast cancer growth, survival, transformation has been well documented in vitro and in animal models (Tab. 1) (4). Reducing ligand availability by excess IGF-BP1 or exposure to suramin blocked IGF-IR activation and limited breast cancer cell proliferation. Furthermore, inhibiting the expression of the IGF-IR with an anti-IGF-IR RNA, or its function with anti-IGF-IR antibodies or dominant-negative mutants, resulted in growth inhibition and reduced transforming potential (4). Our studies with MCF-7 breast cancer cell lines expressing anti-IRS-1 or anti-SHC RNAs (MCF-7/anti-IRS-1 or MCF-7/anti-SHC cells) demonstrated that both IRS-1- and SHC-dependent signals are necessary for cell

proliferation and transformation (22). The critical role of IRS-1 (but not IRS-2) and IRS-1 downstream pathways--Ras/MAP and PI-3K in the growth of estrogen receptor (ER)-positive breast cancer cells has recently been confirmed by Yee laboratory (29). Using dominant-negative IGF-IRs lacking the C-terminus, we demonstrated that in breast tumor cells, like in fibroblasts, the signal originating from the C-terminal portion is essential for transformation in vitro and tumorigenesis in vivo (4).

Dunn et al. has shown that activation of the IGF-IR protects breast cancer cells from apoptosis induced by various therapeutic agents, serum deprivation and irradiation (30). Our results with MCF-7 cells in which IRS-1 has been downregulated by either anti-IRS-1 oligonucleotides, expression of anti-IRS-1 RNA, or antiestrogen treatment suggest that IRS-1/PI-3K signal is required for IGF-IR-induced survival (22, 25, 31).

Amplification of IGF-IR signaling and anchorage-dependent and -independent growth of breast cancer cells. Further understanding of IGF-IR function in breast cancer pathobiology stemmed from studies of cells with amplified IGF-IR signaling. In order to correlate the strength of the IGF signal with the progression towards a more neoplastic phenotype, we developed a series of MCF-7-derived cell lines overexpressing different levels of either the IGF-IR (MCF-7/IGF-IR cells), IRS-1 (MCF-7/IRS-1 cells), or SHC (MCF-7/SHC cells) (25, 28, Surmacz et al., submitted).

Overexpression of the IGF-IR (8-50-fold) was paralleled by enhanced IGF-IR tyrosine kinase activity and hyperphosphorylation of IRS-1, even in the absence of exogenous IGF-I. Compared with the parental cells, all MCF-7/IGF-IR clones exhibited

enhanced autocrine growth in serum-free medium and improved growth responsiveness to low concentrations of IGF (0.1-1.0 ng/ml), especially in the presence of 10 nM estradiol (E2). With higher doses of IGF-I (4-50 ng/ml), the synergistic effect was not seen and the maximal mitogenic effect was achieved with IGF-I alone (28). Similar results were described by Daws et al., who independently developed IGF-IR overexpressing MCF-7 clones (32). Interestingly, we as well as others noticed that high doses of IGF-I (20-50 ng/ml) combined with 10 nM E2 inhibited MCF-7/IGF-IR cell growth, especially in the clones with the highest IGF-IR levels (28, 32).

Anchorage-independent growth of MCF-7/IGF-IR cells treated with E2 was slightly elevated relative to the parental cells, but this effect of IGF-IR overexpression was not present in cells treated with both E2 and IGF-I or cultured in serum-containing medium (28, 32).

In contrast with the modest effects of amplified IGF-IR, overexpression of IRS-1 (1.5-9-fold) produced evident changes in the growth phenotype (25). In MCF-7/IRS-1 cells, proliferation was enhanced under all studied conditions (serum-free and serum-containing medium, or serum-free medium with 20 ng/ml IGF), and the addition of E2 never inhibited the growth. Also, MCF-7/IRS-1 cells exhibited greatly enhanced potential for soft agar growth, especially in the presence of high (200-400 ng/ml) doses of IGF-I. Remarkably, this IGF-dependent transformation was further potentiated with E2. The above effects were correlated with the cellular levels of IRS-1 and the extent of IRS-1 tyrosine phosphorylation (25).

Amplification of SHC in MCF-7 cells (2-7-fold) did not alter growth properties under standard monolayer or anchorage-independent conditions, but it amplified cell-substrate interactions on fibronectin (Surmacz et al. submitted).

IGF-IR/ER cross-talk. In hormone-dependent breast cancer cells, ER and IGF-IR are co-expressed and E2 acts in synergy with IGF-I to stimulate proliferation (4). In part, the effects of E2 are mediated via sensitization of cells to IGF action. E2 treatment up-regulates IGF-IR mRNA and protein levels by 2-10-fold, which is reflected by enhanced IGF-IR tyrosine phosphorylation (4, 33, 34). Furthermore, E2 significantly (2-5-fold) stimulates the expression of IRS-1 in different ER-positive cell lines, and the extent of this stimulation depends on the cellular ER content (34, Surmacz et al., submitted). Of note, E2 action appears to be at least partially specific to IGF-IR/IRS-1 pathway since it does not modulate SHC levels (Surmacz et al., submitted).

Importantly, various antiestrogens such as Tamoxifen (Tam) and its derivatives, droloxifene, and pure antiestrogens ICI 164,384 and ICI 182,780 inhibit IGF-IR-dependent proliferation (4, 31, 35-37). We demonstrated that on the molecular level, anti-IGF-IR action of Tam and ICI 182,780 is accomplished by downregulation of IRS-1/PI-3 kinase signaling (31, 35). Specifically, growth arrest and apoptosis resulting from antiestrogen treatment were associated with continuos suppression of IRS-1 mRNA and protein expression, reflected by reduced IRS-1 tyrosine phosphorylation, decreased IRS-1/PI-3K binding and inhibition of PI-3K activity (31, 35). These anti-IRS-1 effects of ER antagonists were partially reversed in the presence of IGF-I (35).

Antiestrogens also inhibit IGF-IR expression and tyrosine phosphorylation (by 30-50%) but only in the presence of IGF-I (31, 35). In the absence of IGF-I, Tam and ICI 182,780 enhance IGF-IR phosphorylation, which suggest that the drugs may act through modulation of IGF-I-dependent phosphatases. Indeed, the involvement of tyrosine phosphatases LAR and FAP-1 in antiestrogen inhibition of IGF-dependent growth has been demonstrated by Vignon laboratory (38). Interestingly, in different antiestrogen-treated cell lines, SHC expression or signaling were not altered, while SHC tyrosine phosphorylation was increased in Tam- but not in ICI 182,780-arrested cells (31, 35).

Because E2 upregulates IGF-IR signaling, it has been postulated that amplification of the IGF-IR or its key signaling substrates may lead to estrogen-independence. In agreement with this hypothesis, MCF-7/IRS-1 cells exhibited reduced estrogen requirements for growth and transformation, and were not inhibited by E2 alone or in combination with of IGF-I (25). Interestingly, however, such estrogen-independence has not been detected in MCF-7/IGF-IR cells (28, 32). These cells still appear to remain under ER control as their growth is restrained by high doses of E2 in the presence or absence of IGF-I. This suggest the existence of a negative growth regulatory loop which is not operative in MCF-7/IRS-1 cells and may be triggered by hyperactivation of IGF-I signaling pathways not involving IRS-1.

The role of amplified IGF-IR signaling in the development of antiestrogen-resistance is of particular interest. We and others have shown that overexpression of different IGF-IR signaling elements did not affect ER content (25, 28, 32). However, MCF-7/IRS-1 clones with very high IRS-1 levels (9 or 12-fold overexpression in respect

to MCF-7 cells) exhibited resistance to ICI 182,780 , confirming that IRS-1 pathway is an essential target for antiestrogens and suggesting that overexpression of IRS-1 in tumors may hinder antiestrogen therapy (35). Interestingly, in contrast with IRS-1, overexpressed IGF-IRs (50-fold) or SHC (5-fold) did not alter antiestrogen sensitivity in MCF-7 cells (35).

IGF-IR-dependent cell-cell and cell-substrate interactions in breast cancer cells. Breast cancer cells, like other polarized epithelial cells, are governed by cell-cell and cell-substrate interactions. The regulation of these processes by growth factors is now being increasingly recognized. We studied intercellular interactions of MCF-7 cells and their derivatives with modified IGF-IR signaling. We found that overexpression of the IGF-IR greatly enhanced aggregation of cells in three-dimensional (3-D) culture (28). Specifically, when plated on Matrigel, MCF-7/IGF-IR cells formed large spheroids (150-300 um in diameter) surviving or even proliferating for up to 20 days, while the parental MCF-7 cells formed smaller clusters (50 um) which disaggregated and died after 7 days of culture. Similar stimulation of cell-cell adhesion has been described in IGF-I treated MCF-7 and MCF-7/6 cells as well as in MCF-7 cells constitutively secreting IGF-I (4). Our subsequent research demonstrated that enhanced cell-cell adhesion is IGF-I-specific as it cannot be induced by physiologic concentrations of EGF, IGF-II or insulin (4). The mechanism of this phenomenon is presently under investigation and it appears to involve association of the IGF-IR with the adherens junction complex and upregulation of E-cadherin/alpha-catenin/F-actin connections by IGF-IR-mediated signals (4, 28, Surmacz et

al., in preparation). The signals required for cell-cell adhesion depend on IGF-IR tyrosine kinase activity and the presence of the C-terminus (4, Surmacz et al., in preparation). We also observed that cell-cell adhesion is reduced in MCF-7/anti-SHC, but not MCF-7/anti-IRS-1 cells, which points to SHC as a putative mediator of IGF-induced aggregation (22).

The enhanced intercellular connections and improved survival of IGF-IR overexpressing cells may contribute to their tumorigenic activity in vivo. Indeed, when MCF-7/IGF-IR cells were injected into mammary fat pad of nude mice, they formed tumors after 8 weeks, while the parental MCF-7 cells or MCF-7 clones expressing an IGF-IR with a C-terminal truncation were non-tumorigenic (Surmacz et al., in preparation).

Cell-substrate adhesion and migration of epithelial cells is also regulated by IGF-I. For instance, depolarization of MCF-7 and MCF-7/IGF-IR cells and induction of cell migration can be achieved with a 4 h treatment with 50 ng/ml IGF-I. The initial stages of this process are associated with transient dephosphorylation of focal adhesion proteins FAK, paxillin and p130 Cas (23). IGF-IR pathways involved in the regulation of breast cancer cell motility are still quite obscure, but we observed reduced migration of MCF-7 cells with impaired either SHC and PI-3K signaling (22, 23).

Obviously, extracellular cell context may dictate whether cellular response to IGF-I will involve increased cell-cell adhesion or improved migration. The first instance is seen in cells expressing low levels of integrins necessary for attachment to a given ECM substrate, the second case occurs when the cells interact well with a substrate, or produce sufficient amounts of own ECM (23, 28).

IGF-IR signaling in breast tumors. The studies on IGF-IR expression in breast tumors and its correlation with other host or tumor parameters are very limited (4). Moreover, the interpretation of the available data is complicated by the fact that different techniques were used to assess the IGF-IR levels. The most frequently performed IGF-I binding assay is inherently inaccurate due to the interaction of IGF with membrane IGFBPs, which often results in overestimation of the number of IGF-IR (4). To alleviate this problem, recent studies examined the expression of IGF-IR in tumor samples using anti-IGF-IR antibody-based techniques (radioimmunoassay or immunocytochemistry) (39, 41). Despite the differences in experimental approach, the expression of the IGF-IR in breast tumors has been unequivocally established by all large series studies (>100 samples) (4, 42, 43).

Most important, IGF-IR levels have been found elevated (up to 14-fold) in primary breast cancer compared with that in non-malignant tumors or normal epithelium (39-42) (Tab. 1). The mechanism of the common IGF-IR overexpression in breast cancer is not clear, but it does not appear to be associated with IGF-IR gene amplification since this event was reported in only 2% of cases analyzed (44). Recent data indicate that IGF-IR overexpression may be related to de-repression of IGF-IR transcription due to, often occurring in breast tumors, aberrant expression of tumor supressor protein p53 (40). Overexpression of the IGF-IR in tumors has been found associated with hyperactivation of its tyrosine kinase (up to 6-fold), and correlated with radio-resistance and tumor recurrence at the primary site (39-41). High IGF-IR levels in primary tumors have been

reported as predictors of shorter disease-free survival (DFS), but data on the prognostic value of the IGF-IR for overall survival (OS) are conflicting (4, 41).

Importantly, not only the IGF-IR, but also IRS-1 has been found overexpressed in a fraction of primary breast tumors (34, 45). High levels of IRS-1 correlated with shorter DFS in ER-positive tumors (34). The mechanism of IRS-1 overexpression is not known, but it could be associated with E2 or IGF activity since both mitogens are known to stimulate IRS-1 transcription and both can be found (often at superphysiological concentrations) in breast tumors.

The attempts to correlate IGF-IR expression with other host or tumor variables determined a positive link between the IGF-IR and ER status (4, 42, 43). In addition, frequent co-expression of the IGF-IR and IR has been shown (42). Co-expression of these structurally homologous receptors leads to the formation of functional hybrids which bind IGF-I with high affinity, and thereby amplify IGF-I signal (46).

Several large series analyses found no significant correlations between IGF-IR expression and menopausal status (42), body weight (42), tumor size (42, 43), tumor grade (42, 47, 48), histological type (42, 43), node status (42, 43, 47, 49), or EGFR status (47), and the link with the progesterone receptor (PgR) status is uncertain (4, 42, 43, 47-49). However, because most of the above associations were established based on IGF-I binding assays, they should be re-assessed using more accurate techniques of IGF-IR measurement before any firm conclusions can be drawn.

The expression of IRS-1 correlated with ER levels but not with other parameters such as age, tumor size, or PgR status (34, 45). The levels of another IGF-IR substrate,

SHC, in aggressive and more differentiated breast cancer cell lines are similar, but its activity (tyrosine phosphorylation) in cell lines and tumors reflects the levels of oncogenic kinases ERB2 or c-src (50, 51, Surmacz et al. submitted). SHC association with IGF-IR in breast tumors has not been studied.

Unclear role of the IGF-IR in breast cancer progression. The experimental and clinical evidence points to the fact that IGF-IR may be important in early steps of tumor development, promoting cell growth, survival, and resistance to therapeutic treatments. However, the function of the IGF-IR in the later stages, including metastasis, is still obscure.

Especially intriguing is the fact that whereas IGF-IR has been found overexpressed in primary tumors, its levels, like ER levels, appear to undergo reduction during the course of the disease. For instance, Pezzino et al. assessed IGF-IR status in two patient subgroups, representing either a low risk (ER- and PgR-positive, low mitotic index, diploid) or a high risk (ER- and PgR-negative, high mitotic index, aneuploid) population and found a highly significant correlation between IGF-IR expression and better prognosis (42). Similar conclusions reached Peyratt and Bonneterre (43). Therefore, it has been proposed that like the ER, the IGF-IR marks more differentiated tumors with better clinical outcome. However, it has also been argued that the IGF-IR may play a role in early steps of tumor spread since node-positive/IGF-IR-positive tumors appeared to bear worse prognosis than node-negative/ IGF-IR-positive tumors (47). In addition, quite

rare cases of ER-negative but IGF-IR-positive tumors are associated with shorter DFS (46).

In breast cancer cell lines, hormone-dependence and less aggressive phenotype correlates with a good expression of the IGF-IR and IRS-1 (28, 34). In contrast, highly metastatic ER-negative breast cancer cell lines express low levels of the IGF-IR and often do not respond to IGF-I with growth. Similarly, IRS-1 levels are downregulated in a majority of these cell lines (34, 52). Despite this "IGF-IR-reduced phenotype", metastatic cell lines appear to depend on the IGF-IR. For instance, blockade of the IGF-IR in MDA-MB-231 cells by anti-IGF-IR antibody reduced migration in vitro and tumorigenesis in vivo, and expression of a soluble IGF-IR in MDA-MB-435 cells impaired growth, tumorigenesis and metastasis in animal model (53-55). Whether this particular IGF-I-dependence of metastatic breast cancer cells relates to the survival function of the IGF-IR is under investigation in our laboratory.

Conclusions and Perspectives. Over the past few years much has been learned about the function of the IGF-IR in the process of tumorigenesis. Clearly, IGF-IR-mediated survival and transformation are key factors affecting tumor development. In primary breast cancer, high levels of the IGF-IR may promote survival and proliferation, counteracting cytotoxic or cytostatic effects of drugs or radiation. The mechanism of this IGF-I action includes strengthening intercellular connections, amplification of anti-apoptotic signals, and sensitization of cells to low concentrations of IGFs and E2.

Therefore, targeting the IGF-IR, especially the IGF-IR/IRS-1 pathway, should help eradicating primary tumor cells.

The importance of the IGF-IR in the metastatic breast disease is still not clear. It is possible that the IGF-IR has a role in cell spread, functioning primarily as an anti-apoptotic, and possibly a motogenic factor. Unquestionably, further understanding of IGF-IR function in metastatic cells will be critical in creating successful anti-IGF-IR therapies for late stages of breast cancer.

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Abbreviations: 3-D, three-dimensional; aa, amino acid; DFS, disease-free survival; E-cad, E-cadherin; E2, 17-beta estradiol; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; ER, estrogen receptor; His, histidine; IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; IR, insulin receptor; IRS, insulin receptor substrate; Lys, lysine; OS, overall survival; PI-3K, phosphatidylinositol-3 kinase; PgR; progesterone receptor; PKC, protein kinase C; PTB, phosphotyrosine binding domain; MCF-7/anti-IRS-1 and MCF-7/anti-SHC, MCF-7 cells expressing anti-IRS-1 and anti-SHC RNA, respectively; MCF-7/IGF-IR, MCF-7/IRS-1, and MCF-7/SHC, MCF-7 cells overexpressing the IGF-IR, IRS-1, and SHC, respectively; Ser, serine; SH2, src-homology 2 domain; SHC, src/collagen homology proteins; Tam, Tamoxifen.

Tables

Table 1. IGF-IR function in breast cancer

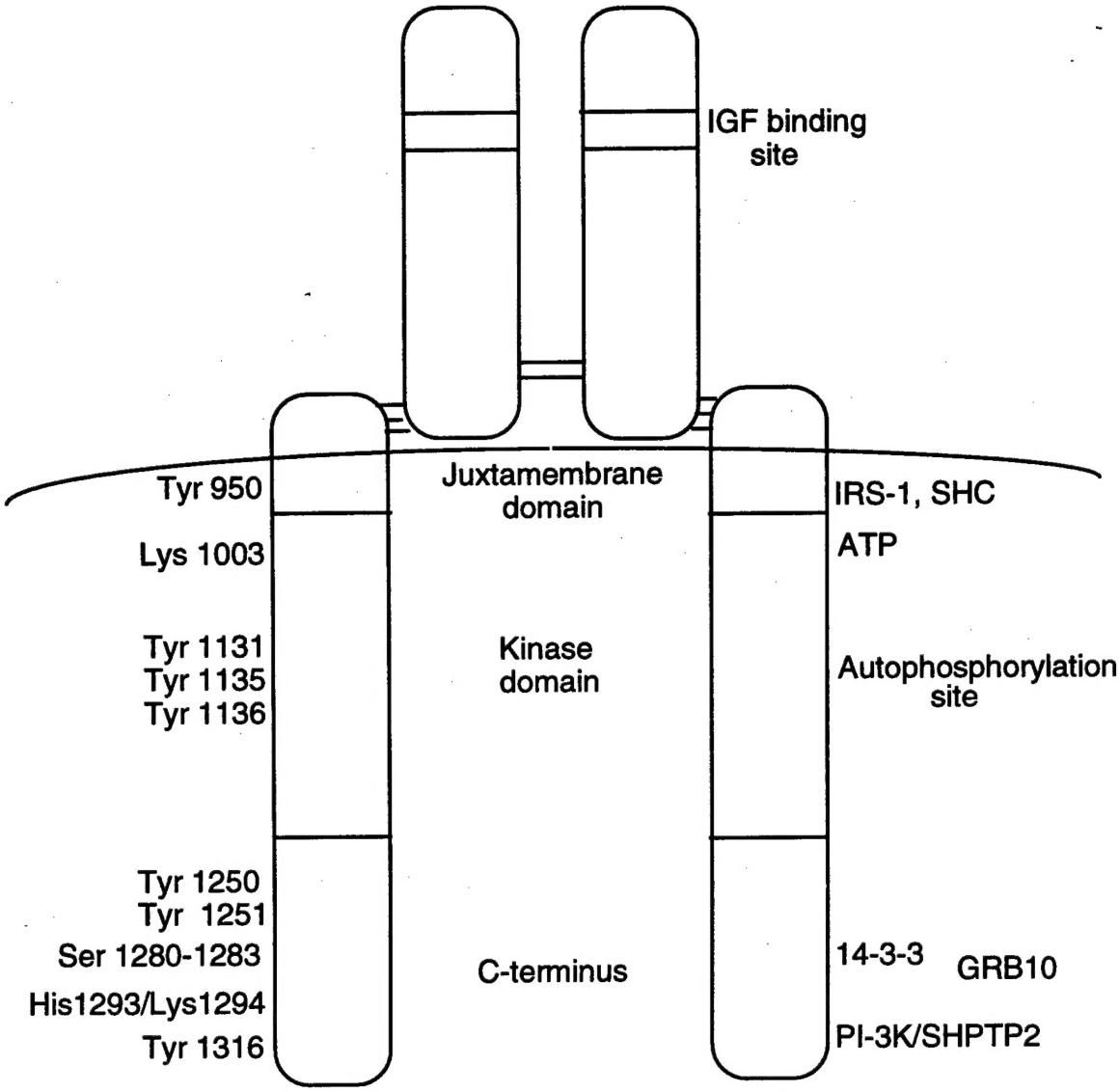
Signaling Molecule	Function in Breast Cancer	
	Experimental Models	Tumors
IGF-IR	elevated in ER-positive breast cancer cells; stimulates proliferation; counteracts apoptotic effects of anti-tumor drugs; improves 3-D growth and survival; regulates cell-substrate connections; required for anchorage-independent growth in vitro and tumorigenesis and metastasis in animal models.	correlates with the ER status; elevated in primary tumors; high levels correlate with radio-resistance and recurrence at the primary site; usually co-expressed with markers of better overall prognosis.
IRS-1	elevated in ER-positive breast cancer cells; required for anchorage-dependent and independent growth; critical for survival; high levels induce estrogen-independence and antiestrogen-resistance.	correlates with shorter DFS in ER-positive primary tumors.
SHC	required for proliferation, anchorage-independent growth, migration, and cell-cell adhesion; high levels improve adhesion to fibronectin.	?

References in the text.

Legend to Figures

Fig. 1. Structure of the IGF-IR. The key residues involved in IGF-IR signaling are indicated on the left; the signaling elements binding to these regions of the IGF-IR are listed on the right.

Figure 1


IGF binding site
Tyr 950
Juxtamembrane domain
IRS-1, SHC
Lys 1003
ATP
Tyr 1131
Tyr 1135
Tyr 1136
Kinase domain
Autophosphorylation site
Tyr 1250
Tyr 1251
Ser 1280-1283
His1293/Lys1294
Tyr 1316
C-terminus
14-3-3
GRB10
PI-3K/SHPTP2